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FORM PTO-1390 (REV. 11-94)	U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE	ATTORNEY'S DOCKET NUMBER
TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US)		8484-095-999
		09/743953

INTERNATIONAL APPLICATION NO.	INTERNATIONAL FILING DATE	PRIORITY DATE CLAIMED
PCT/DE99/02185	13 July 1999	13 July 1998

TITLE OF INVENTION

INHIBITION OF ALOPECIA

APPLICANT(S) FOR DO/EO/US

Thomas BOEHM, Thomas SCHLAKE, and Natalia MEIER

Applicant herewith submits to the United States Designated/ Elected Office (DO/EO/US) the following items under 35 U.S.C.

1. ☒ This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.

2. ☐ This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.

3. ☒ This is an express request to begin national examination procedures (35 U.S.C. 371(f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. 371(b) and PCT Articles 22 and 39(1).

4. ☒ A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date.

5. ☒ A copy of the International Application as filed (35 U.S.C. 371(c)(2))
   a. ☒ is transmitted herewith (required only if not transmitted by the international Bureau).
   b. ☐ has been transmitted by the International Bureau.
   c. ☐ is not required, as the application was filed in the United States Receiving Office (RO/US)

6. ☒ A translation of the International Application into English (35 U.S.C. 371(c)(2)).

7. ☒ Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3))
   a. ☒ are transmitted herewith (required only if not transmitted by the International Bureau).
   b. ☐ have been transmitted by the International Bureaus.
   c. ☐ have not been made; however, the time limit for making such amendments has NOT expired.
   d. ☐ have not been made and will not be made.

8. ☒ A translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 37(c)(3)).

9. ☒ An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)) (unexecuted).

10. ☐ A translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).

Items 11. to 16. below concern document(s) or information included:

11. ☐ An Information Disclosure Statement under 37 CFR 1.97 and 1.98.

12. ☐ An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.

13. ☒ A FIRST preliminary amendment.
    ☐ A SECOND or SUBSEQUENT preliminary amendment.

14. ☒ A substitute specification and marked-up version thereof comparing the substitute specification to the English translation of the priority application.

15. ☐ A change of power of attorney and/or address letter.

16. ☒ Other items or information:

Return Post Card;
Copies of:
- Request for International Application;
- Copy of front page of published PCT Application;
- International Search Report;
- Request for Preliminary Examination;
- Preliminary Examination Report.

ONAL APPLICATION NO.
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13 July 1999

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7. ☒ The U.S. National Fee (35 U.S.C. 371(c)(1)) and other fees as follows:

CLAIMS				
(1)FOR	(2)NUMBER FILED	(3)NUMBER EXTRA	(4)RATE	(5)CALCULATIONS
TOTAL CLAIMS	19 - 20	0	X $ 18.00	$ 0.00
INDEPENDENT CLAIMS	2 - 3	0	X $ 80.00	0.00
MULTIPLE DEPENDENT CLAIM(S) (if applicable)			+ $ 270.00	$ 260.00

BASIC NATIONAL FEE (37 CFR 1.492(a)(1)-(5)):
CHECK ONE BOX ONLY

- ☐ International preliminary examination fee paid to USPTO (37 CFR 1.482) ..... $ 690
- ☐ No international preliminary examination fee paid to USPTO (37 CFR 1.482) but international search fee paid to USPTO (37 CFR 1.445(a)(2)) ..... $ 710
- ☐ Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO $1000
- ☐ International preliminary examination fee paid to USPTO (37 CFR 1.482) and all claims satisfied provisions of PCT Article 33(2) to (4) ..... $ 100
- ☒ Filing with EPO or JPO search report ..... $ 860

Surcharge of $130.00 for furnishing the National fee or oath or declaration later than 20 30 mos. from the earliest claimed priority date (37 CFR 1.492(e)).	
TOTAL OF ABOVE CALCULATIONS	= 1,120.00
Reduction by 1/2 for filing by small entity, if applicable. Affidavit must be filed also. (Note 37 CFR 1.9, 1.27, 1.28).	- $ 560.00
SUBTOTAL	= 560.00
Processing fee of $130.00 for furnishing the English Translation later than 20 30 mos. from the earliest claimed priority date (37 CFR 1.492(f)).	+
0 TOTAL FEES ENCLOSED	$ 560.00

a. ☐ A check in the amount of $__ to cover the above fees is enclosed.

b. ☒ Please charge Deposit Account No. 16-1150 in the amount of $ 560.00 to cover the above fees. A copy of this sheet is enclosed.

c. ☒ The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. 16-1150 (order no. 8484-095-999). A copy of this sheet is enclosed.

8. ☐ Other instructions
n/a

9. ☒ All correspondence for this application should be mailed to
PENNIE & EDMONDS LLP
1155 AVENUE OF THE AMERICAS
NEW YORK, NEW YORK 10036-2711

10. ☒ All telephone inquiries should be made to (212) 790-2803

git Millauer			
ME	SIGNATURE	43,341 REGISTRATION NUMBER	12 January 2001 DATE
Laura A. Coruzzi			
: Laura A. Coruzzi			
g. No. 30,742)			

Express Mail No.: EL 451 595 865 US

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Application of: Boehm *et al.*

Serial No.: To be assigned

Group Art Unit: To be assigned

Filed: Herewith

Examiner: To be assigned

For: **INHIBITION OF ALOPECIA**

Attorney Docket No.:
8484-095-999

PRELIMINARY AMENDMENT

Assistant Commissioner for Patents
Washington, D.C. 20231

Sir:

In accordance with Rule 111 of the Rules of Practice, 37 C.F.R. § 1.111, please consider and enter the following amendments and remarks.

AMENDMENTS

IN THE SPECIFICATION:

Please amend the specification as follows:

Please replace the specification as filed in PCT/DE99/02185 by the enclosed Substitute Specification under 37 C.F.R. § 1.125. The Substitute Specification has been prepared solely for the purpose of complying with the rules of practice; it does not introduce new matter. A marked-up copy of the Substitute Specification showing any matter being added and any matter being deleted from the original specification is enclosed in accordance with 37 C.F.R. § 1.125(b)(2).

IN THE CLAIMS:

Please amend the claims as follows:

1. (Amended) A process for inhibiting alopecia, comprising:

_____[the increase] increasing in the cellular amount of hair [keratins] keratin.

2. (Amended) The process [according to claim] of Claim 1, wherein said hair [keratins are] keratin is added to the cells.

3. (Amended) The process [according to claim] of Claim 2, wherein [the] said hair [keratins are] keratin is present in the form of [DNA expressing] a polynucleotide encoding the same.

4. (Amended) The process [according to any one of claims] of Claim 1 [to 3], wherein [the gene expression of substances] a substance activating gene expression in hair [keratins are] keratin is added to the cells.

5. (Amended) The process [according to claim] of Claim 4, wherein [the] said substances are present in the form of [DNA expressing] a polynucleotide encoding the same.

6. (Amended) The process [according to any one of claims] of Claim 1 [to 5], wherein [the] said hair [keratins comprise] keratin is selected from the group consisting of Ha2, Ha2, Ha3 and Ha4.

7. (Amended) The process [according to claim] of Claim 4 or 5, wherein [the substances comprise] said substance is selected from the group consisting of the gene product of the whn gene [and/or] and a substance [the expression of substances] activating the expression of the whn gene.

8. (Amended) A process of identifying alopecia-inhibiting substances, [in which the] comprising:

(a) cultivating cells in the presence of a candidate substance;

(B) determining an increase in the cellular amount [is determined by] of hair [keratins] keratin [and/or substances] or of a substance activating the gene expression [thereof] of hair keratin.

9. (Amended) The process [according to claim] of Claim 8, wherein said cells [are used in which] comprise a fusion gene, wherein one or several [expressing] hair keratin expressing genes [are present in] are fused [form with] to a reporter gene.

10. (Amended) The process [according to claim] of Claim 8 or 9, wherein [the] said hair [keratins comprise] keratin is selected from the group consisting of Ha1, Ha2, Ha3, and Ha4.

11. (Amended) The process [according to any one of claims] of Claim 8 [to 10], wherein said cells [are used in which] comprise a fusion gene, wherein one or several [expressible substances] genes expressing a substance activating the gene expression of hair [keratins are] keratin [are present in] are fused [form] [with the] to a reporter gene.

12. (Amended) The process [according to any one of claims] of Claim 8 [to 11], wherein [the substance comprise] said substance is a gene product of the whn gene.

13. (Amended) The process [according to any one of claims] of Claim 9 [to 12], wherein [the] said reporter gene [codes for] encodes an enzyme.

14. (Amended) The process [according to any one of claims] of Claim 9 [to 12], wherein [the] said reporter gene [codes for] encodes a fluorescent protein.

15. (Amended) The process [according to any one of claims] of Claim 9 [to 14], wherein [the] said fusion [genes are] gene is present in extrachromosomal form.

16. (Amended) The process [according to any one of claims] of Claim 9 [to 14], wherein [the] said fusion [genes are] gene is integrated in the cell genome.

17. (Amended) The process [according to any one of claims] of Claim 9 [to 16], [which also uses substances for the detection of the expressed] further comprising detecting expression of hair [keratins and/or] keratin, or of [substances] a substance activating the gene expression [thereof and/or the] of hair keratin or said fusion [genes] gene by use of a suitable substance.

REMARKS

Claims 1-17 are pending in this application. The claims as pending are attached hereto as *Appendix A*.

The above amendments do not introduce new matter, and they are fully supported by the specification of the subject application and the claims as originally filed.

Applicants respectfully request that the above-made amendments be made of record in the file history of the instant application.

Respectfully submitted,

Date January 12, 2001

43,341
Birgit Millauer (Reg. No.)

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APPENDIX A
Claims as Pending
8484-095-999

1. A process for inhibiting alopecia, comprising:

increasing in the cellular amount of hair keratin.

2. The process of Claim 1, wherein said hair keratin is added to the cells.

3. The process of Claim 2, wherein said hair keratin is present in the form of a polynucleotide encoding the same.

4. The process of Claim 1, wherein a substance activating gene expression in hair keratin is added to the cells.

5. The process of Claim 4, wherein said substances are present in the form of a polynucleotide encoding the same.

6. The process of Claim 1, wherein said hair keratin is selected from the group consisting of Ha2, Ha2, Ha3 and Ha4.

7. The process of Claim 4 or 5, wherein said substance is selected from the group consisting of the gene product of the whn gene and a substance activating the expression of the whn gene.

8. A process of identifying alopecia-inhibiting substances, comprising:

(a) cultivating cells in the presence of a candidate substance;

(B) determining an increase in the cellular amount of hair keratin or of a substance activating the gene expression of hair keratin.

9. The process of Claim 8, wherein said cells comprise a fusion gene, wherein one or several hair keratin expressing genes are fused to a reporter gene.

10. The process of Claim 8 or 9, wherein said hair keratin is selected from the group consisting of Ha1, Ha2, Ha3, and Ha4.

11. The process of Claim 8, wherein said cells comprise a fusion gene, wherein one or several genes expressing a substance activating the gene expression of hair keratin are fused to a reporter gene.

12. The process of Claim 8, wherein said substance is a gene product of the whn gene.

13. The process of Claim 9, wherein said reporter gene encodes an enzyme.

14. The process of Claim 9, wherein said reporter gene encodes a fluorescent protein.

15. The process of Claim 9, wherein said fusion gene is present in extrachromosomal form.

16. The process of Claim 9, wherein said fusion gene is integrated in the cell genome.

17. The process of Claim 9, further comprising detecting expression of hair keratin, or of a substance activating the gene expression of hair keratin or said fusion gene by use of a suitable substance.

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

INHIBITION OF ALOPECIA

This is a national phase filing of the Application No. PCT/DE99/02185, which was filed with the Patent Corporation Treaty on 13 July 1999, and is entitled to priority of the German Patent Application 198 31 043.9, filed 13 July 1998.

I. FIELD OF THE INVENTION

The present invention relates to a process for inhibiting alopecia and a system of identifying alopecia-inhibiting substances.

II. BACKGROUND OF THE INVENTION

Alopecia is a wide-spread hair disease which may result in the complete loss of the hair. The causes of alopecia are not known. In so far it is not possible to influence this disease in well-calculated fashion.

Therefore, it is the object of the present invention to provide a product by means of which this can be achieved.

According to the invention, this is achieved by the subject matters defined in the claims.

III. SUMMARY OF THE INVENTION

The present invention relates to a process for inhibiting alopecia, comprising the increase in the cellular amounts of hair keratins and a system of identifying substances inhibiting alopecia.

IV. BRIEF DESCRIPTION OF THE DRAWINGS

FIGURE 1 shows an *in situ* RNA hybridization using a probe for mHa3 in normal (whn + / +) and mutant (whn - / -) mice. The transcripts for mHa3 (perceptible as brown

silver grains) cannot be detected in hair follicles of nude mice. The line corresponds to 100 μm.

FIGURE 2 shows the expression of whn and hair keratins in the hair follicle of a mouse.

FIGURE 2A: Northern filter hybridization with RNA from the total skin of normal mice (whn + / +) and nude mice (whn - / -) by means of probes for hprt and whn genes as well as Ha1, Ha3, Ha4 genes at three times following the birth dP7, 7 days after the birth, etc.).

FIGURE 2B: *In situ* RNA hybridization in the skin from normal (whn + / +) and nude mice (whn - / -) with probes for Ha1, Ha3, and Ha4 genes. An autoradiogram of skin cuts on day 7 afer the birth is shown.

FIGURE 3 shows the control of keratin gene expression. HeLa cells were transiently transfected with a whn expression construct (+), and the presence of Ha3-specific mRNA was detected by means of RT-PCR. The molecular weight markers are given in bp.

V. DETAILED DESCRIPTION OF THE INVENTION

It is the object of the present invention to provide a product by means of which this can be achieved.

The present invention is based on the applicant's findings that certain forms of alopecia are based on an unbalanced keratinization of the hair. Furthermore, he has found that in the case of alopecia the mRNA of various genes is lacking, *e.g.,* that of the Ha3 gene, or underrepresented, *e.g.,* those of Ha1, Ha2, and Ha4 genes (FIGURES 1 and 2). The gene products of Ha1, Ha2, Ha3, and Ha4 genes are hair keratins. The applicant has found that the expression of the Ha3 gene is controlled by a gene product of the whn gene. In particular, he has found that the expression of the Ha2 gene can be induced by the expression of the whn gene (FIGURE 3). He has also found that the expression of other hair keratin genes is essentially influenced by the gene product of the whn gene. The applicant has also found that the expression of the whn gene varies in the course of the hair cycle. In particular, he has found that the whn expression in the telogen of the hair cycle drops to no longer detectable levels. In addition, he has discovered that the whn gene can be

transcribed by two promoters. The applicant has obtained his findings by means of nude mice and HeLa cells.

According to the invention the applicant's findings are used for a process for inhibiting alopecia, which comprises the increase in the cellular amount of hair keratins.

The expression "increase in the cellular amount of hair keratins" refers to the fact that the amount of one or several hair keratins, particularly of Ha1, H2, Ha3 and Ha4, which may be present in small amount or not al all, is increased in cells. This can be achieved by common methods and substances, respectively. For example, one or several hair keratins, particularly Ha1, Ha2, Ha3 and Ha4, may be added to the cells as such or in the form of DNA encoding the same. The DNA may be present in common expression vectors. It is also possible to add substances which activate the expression of one or several hair keratins, particularly of Ha1, Ha2, Ha3 and Ha4. Such substances are, *e.g.,* the gene product of the whn gene or a DNA encoding the same. It may be present in common expression vectors. Moreover, substances may be added which activate the expression of the whn gene. They may also be present as such or in the form of DNA encoding the same, it being possible for the latter to be also present in common expression vectors. The expression "cells" comprises cells of any kind and origin. In addition, it comprises tissues and organisms, particularly animals and human beings.

Substances inhibiting alopecia can be administered as usual, preferably locally. The substances may also be present in common formulations. If the substances are administered locally, *e.g.,* creams, ointments, shampoos and hair tonics will be suitable. The substances may also be present as particles which are easily absorbed. Examples of such particles are liposomes. A person skilled in the art knows processes to discover the suitable formulations and forms of administration, respectively, for the individual substances.

A further subject matter of the present invention relates to a system of identifying substances which are suited to inhibit alopecia. Such a system comprises the increase in the cellular amount of hair keratins and/or substances activating the gene expression thereof. In particular, the system comprises animals or cells, cells being preferred, in which one or several expressible hair keratin genes and/or one or several expressible genes, whose gene products activate the gene expression of hair keratins, are present each in fused form with a reporter gene. The hair keratin genes may be particularly those of Ha1, Ha2, Ha3 and Ha4.

Moreover, it is favorable for the substance activating the gene expression of hair keratins to be a gene product of the whn gene. In addition, the above genes may have a wild type sequence or a modified sequence, it being possible for the latter to differ from the wild type sequence by one or several base pairs. The differences may exist in the form of additions, deletions, substitutions and/or inversions of base pairs. Besides, an above reporter gene may be any gene, particularly it may code for an enzyme, *e.g.,* alkaline phosphatase, or a fluorescent protein, *e.g.,* GFP. The fusion genes may also be available in extrachromosomal fashion or in the cell genome, particularly in place of one or both alleles of hair keratins and/or the genes whose gene products activate the expression of hair keratins. Besides, the system may contain substances which are suited to detect the expressed hair keratins and/or substances activating the gene expression thereof and the fusion genes, respectively. Such substances may be suited for the detection on a nucleic acid level and protein level, respectively.

By means of the present invention it is possible to inhibit alopecia. It is also possible to diagnose alopecia by determining, *e.g.,* the gene expression of hair keratins and/or substances which activate it. Moreover, it is possible to discover substances which are adapted to inhibit alopecia. For this purpose, a system is provided which is suited for the rapid and reliable screening of the most varying substances. Thus, the present invention provides products serving for diagnosing and treating a wide-spread hair disease.

The below examples explain the invention in more detail. The following preparations and examples are given to enable those skilled in the art to more clearly understand and to practice the present invention. The present invention, however, is not limited in scope by the exemplified embodiments, which are intended as illustrations of single aspects of the invention only, and methods which are functionally equivalent are within the scope of the invention. Indeed, various modifications of the invention in addition to those described herein will become apparent to those skilled in the art from the foregoing description and accompanying drawings. Such modifications are intended to fall within the scope of the appended claims.

VI. EXAMPLES

A. Example 1: Detection of the Loss of Expression of the Ha3 Gene in Mice Suffering from Alopecia

The "Representational Difference Analysis" (RDA) method was carried out. This method comprises the isolation of mRNA from skin cells of (whn + / +) mice and (whn - / -) mice (mice suffering from alopecia and having no expression of the whn gene), respectively, the transcription of mRNA into cDNA, and the differentiation of the cDNA, thereby identifying the one underexpressed and overexpressed, respectively, in (whn - / -) mice.

A) Sequence of the Oligonucleotide Adapters

The following oligonucleotide adapter pairs were required for RDA:

R-Bgl-12: 5' -GATCTGCGGTGA-3'

R-Bgl-24: 5' -AGCACTCTCCAGCCTCTCACCGCA-3'

R-Bgl-12: 5' -GATCTGTTCATG-3'

R-Bgl-24: 5' -ACCGACGTCGACTATCCATGAACA-3'

R-Bgl-12: 5' -GATCTTCCCTCG-3'

R-Bgl-24: 5' -AGGCAACTGTGCTATCCGAGGGAA-3'

B) Production of Poly A-mRna from Tissues to Be Compared with One Another

First, RNA was obtained from the skin of (whn + / +) mice and (whn - / -) mice, respectively, according to the "single step RNA extraction" method (Chomczynski and Sacchi, 1987). The poly A-mRNA fractions from both RNA populations were then isolated by means of dynabeads oligo(dT) according to the corresponding protocol from the company of Dynal.

C) Synthesis of Double-stranded cDNA

The "ribo clone DNA synthesis kit" from the company of Promega was used for the synthesis of double-stranded (whn + / +) cDNA and (whn - / -) cDNA, respectively. 4 μg poly A-mRNA were used each to obtain about 2 μg cDNA.

D) Difference Analysis

1. Restriction digestion of the double-stranded cDNAs.

   a) About 2 μg of each cDNA were digested in a 100 μl reaction batch by the restriction endonuclease DphII at 37°C for 2 h.

   b) The reaction solutions were then extracted twice using a phenol/chloroform mixture (1:1) and once using 100% chloroform.

   c) The DNA included in the aqueous phases of the two reaction batches was mixed with 2 μg glycogen, 50 μl 10 M ammonium acetate, and 650 μl 100% ethanol each and precipitated on ice for 20 min. Following 14 minutes of centrifugation at 4°C and 14,000 rpm, the supernatant was discarded and the DNA pellet was washed with 70% ethanol. After another centrifugation and removal of the alcoholic phase, the dried DNA was resuspened in 20 μl TE buffer.

2. Litigation of the cDNAs to the R-Bgl oligonucleotide adapter pair

   a) A reaction vessel collected the following:

   20 μl cut cDNA (total reaction batch from item D)1c)
   8 μg R-Bgl-24
   4 μg R-Bgl-12
   6 μl 10 x ligase buffer
   x μl water
   57 μl final volume

b) The reaction batch was heated in a thermocycler (Peltier Thermocycler PTC-200, MJ Research) to 50°C, kept at this temperature for 1 min., and then cooled again down to 10°C in the course of one hour (ramp rate: 0.1°C/9 sec).

c) After adding 3 μl T4 DNA ligase (1 U/μl), the mixture was incubated at 16°C overnight.

3. Synthesis of "representations" of the cDNA populations to be compared with one another

a) In order to generate what is called "representations" of the ligated cDNAs, the volume of the ligation batches from item 2c) was initially completed by adding 140 μl water each to give 200 μl.
Then, 30 reactions of 200 μl each were prepared from this dilute solution per cDNA population (whn + / +) skin and (whn - / -) skin.
The following reactants were added to such a batch one after the other:
143 μl water
20 μl 10x PCR buffer
20 μl 2 nM dNTPs
20 μl 25 nM Mg chloride
2 μl R-Bgl-24 (1 μg/μl)
4 μl dilute ligation batch

b) PCR:
3 min.: 72 °C
addition of 1 μl Taq-DNA polymerase (5 U/μl)
20 x: 5 min. : 95°C
3 min.: 72°C
finally: cooling to 4°C.

c) For preparing the reaction solutions, 4 reaction batches each were collected in a vessel.

Extraction:	2 x with 700 µl phenol/chloroform each (1:1), 1 x with chloroform 100 %;
Precipitation:	addition of 75 µl 3 M Na acetate solution (pH 5.3) and 800 µl 2-propanol to each reaction vessel, 20 min. on ice.
Centrifugation:	14 min., 14,000 rpm, 4°C.

Washing of the DNA pellets with ethanol 70% and resuspension in such an amount of water that a concentration of 0.5 µg/µl resulted.

4. Restriction digestion of the "representations"

a) In order to remove the R-Bgl oligonucleotide adapters, 300 µg of each representation (whn + / +) skin and (whn - / -) skin, repetitively, were subjected to restriction digestion. After adding the following reactants, incubation was carried out at 37°C for 4 hours:

600 µl cDNA representation (0.5 µg/µl)
140 µl 10 x DpnII buffer
100 µl DpnII (10 U/µl)
560 µl water.

b) The restriction digestion batch was distributed to 2 vessels prior to its preparation.

Extraction:	2 x phenol/chloroform (1:1), 1 x chloroform 100 %;
Precipitating:	addition of 70 µl 3 M Na acetate (pH 5.3), 700 µl 2-propanol to each vessel, 20 min. on ice.
Centrifugation:	14 min., 14,000 rpm, 4°C.

The DNA pellet was washed with ethanol 70% and resuspended in such an amount of water that a concentration of 0.5 µg/µl resulted.

The resulting DpnII-digested (whn + / +) skin cDNA representation represented the driver DNA population to be used for the subtractive hybridization.

5. Synthesis of the tester DNA population

a) 20 μg of the (whn - / -) skin cDNA representation digested by DpnII (= tester DNA) was separated in a TAE gel by means of electrophoresis:

40 μl tester DNA (0.5 μg/μl)
50 μl Te buffer
10 μl 10 x loading buffer

were placed on a 1.2% agarose TAE gel. A voltage was applied to the gel until the bromophenol blue component of the loading buffer had migrated about 2 cm.

b) Thereafter, the bands containing the representation DNA were cut out off the gel and eluted by means of the "agarose gel DNA extraction kit" from the company of Boehringer Mannheim.
The DNA extracts were collected, so that a total of 60 μl solution was obtained. The concentration of this solution was evaluated by electrophoresis of 5 μl in a 1% agarose gel.

c) Eventually, the tester DNA was ligated with the J-oligonucleotide pair:

2 μg tester DNA eluate
6 μl 10 x ligase buffer
4 μl J-Bgl-24 (2 μg/μl)
4 μl J-Bgl-12 (1 μg/μl)
x μl water
57 μl final volume

d) Transferring the reaction batch to the thermocycler:
1 min.: 50°C
cooling down to 10°C within 1 h (ramp rate: 0.1°C/9 sec.)

e) The addition of 3 µl T4 DNA ligase (1U/µl) was followed by incubation at 16°C overnight.

f) Adjustment of the concentration of the tester DNA to about 10 ng/µl by the addition of 120 µl water.

6. Subtractive hybridization

a) 80 µl driver DNA (40 µg) from step 4 and 40 µl (0.4 µg) dilute tester DNA from step 5., ligated with J-oligonucleotides, were collected in a reaction vessel and extracted 2 x with phenol/chloroform (1:1) and once with chloroform 100%.

b) Precipitation by adding 30 µl 10 M ammonium acetate, 380 µl ethanol 100%, -70°C for 10 min.

Centrifugation:	14 min., 14,000 rpm, 4°C
Thereafter:	2 x washing the pellet with ethanol 70%, short centrifugation after each wash step; drying of the DNA pellet.

c) The DNA was resuspended in 4 µl EE x3 buffer (30 mM EPPS, pH 8.0 at 20°C (company of Sigma), 3 mM EDTA) - with pipetting off and on for about 2 min., then heated to 37°C for 5 min., shortly vortexed and eventually the solution was collected again at the vessel bottom by centrifugation. Finally, the solution was coated with 35 µl of mineral oil.

d) Transferring the reaction batch to the thermocycler:

5 min.: 98°C, cooling down to 67°C and immediate addition of 1 µl 5 M NaCl to the DNA, incubation at 67°C for 20 h.

7. Synthesis of the first difference product

a) After removing the mineral oil as completely as possible, the DNA was diluted step-wise:

(1) addition of 8 µl TE (+ 5 µg/µl yeast RNA),

(2) addition of 25 µl TE - thereafter thorough mixing,

(3) addition of 362 µl TE - vortex.

b) 4 PCRs were prepared for each subtractive hybridization. Per reaction:

127 µl water

20 µl 10 x buffer

20 µl 2 mM dNTPs

5 µl 25 mM Mg chloride

20 µl dilute hybridization solution (from step 7a))

c) PCR program:

3 min.: 72°C

addition of 1 µl Taq DNA polymerase (5 U/µl)

5 min.: 72°C

addition of 2 µl primer J-Bgl-24 (1 µg/µl)

10 x: 1 min.: 95°C

3 min.: 70°C

finally: 10 min.: 72°C, then cooling down to room temperature.

d) The 4 reaction batches were collected in a 1.5 ml vessel.

Extraction: 2 x phenol/chloroform (1:1), 1 x chloroform 100%.

After the addition of 2 µg glycogen carrier:

Precipitation with 75 µl 3 M Na acetate (pH 5.3), 800 µl 2-propanol, 20 min. on ice.

Centrifugation: 14 min., 14,000 rpm, 4°C.
Washing of the DNA pellet with ethanol 70%.
After drying the DNA, resuspension in 40 µl water.

e) 20 µl of the resuspended DNA from d) were subjected to mung bean nuclease digestion (+ MBN):
20 µl DNA
4 µl 10 x mung bean nuclease buffer (company of NEB)
14 µl water
2 µl mung bean nuclease (10 U/µl; company of NEB)
35 min., 30°C.
The reaction was stopped by adding 160 µl of 50 mM Tris-HCl (ph 8.9) and 5 minutes of incubation at 98°C. Thereafter, the vessel was placed on ice up to the next step.

f) During the MBN incubation, 4 further PCRs were prepared on ice:
127 µl water
20 µl 2 mM dNTPs
10 µl 25 mM Mg chloride
2 µl J-Bgl-24 (1 µg/µl)
20 µl MBN-digested DNA.

g) PCR program:
1 min.: 95°C
allowing to cool down to 80°C, addition of 1 µl Taq DNA polymerase (5 U/µl)
18 x: 1 min.: 95°C
3 min.: 70°C
finally: 10 min.: 72°C, allowing to cool down to 4°C.

h) The 4 PCR batches were collected in a vessel

Extraction: 2 x phenol/chloroform (1:1)
1 x chloroform 100%.

Precipitation: 75 µl 3 M Na acetate (pH 5.3), 800 µl 2-propanol, 20 min. on ice.

Centrifugation: 14 min., 14,000 rpm, 4°C.

Washing of the DNA pellet with ethanol 70%.

Resuspension of the DNA in 100 µl water (resulting concentration: 0.5 µg/µl); the resulting solution represented the first difference product.

8. Exchange of the oligonucleotide adapters of the difference product.

a) Removal of the oligonucleotide adapters by restriction digestion using DpnII:

40 µl difference product 1 (0.5 µg/µl)
30 µl 10 x DpnII buffer
15 µl DpnII (U/µl)
215 µl water
37°C for 2 h.

b) Preparation of the reaction batch:

Extraction: 2 x phenol/chloroform (1:1), 1 x chloroform 100%.

Precipitation: 33 µl 3 M Na acetate (pH 5.3), 800 µl ethanol 100%, -20°C for 20 min.

Centrifugation: 14 min., 14,000 rpm, 4°C.

Washing of the pellet in ethanol 70% and resuspension in 40 µl water.

c) Ligation of the difference product to N-Bgl oligonucleotide adapter pair 1 µl of the prepared DNA solution from step b) was diluted with

9 μl water to give a concentration of 50 ng/μl; 4 μl of this solution were used in the following reaction:
4 μl DpnII-digested difference product 1 (200 ng)
6 μl 10 x ligase buffer
2.5 μl N-Bgl-12 (2 μg/μl)
42.5 μl water.

d) After transferring the reaction batch to the thermocycler:
1 min.: 50°C,
allowing to cool down to 10°C within one hour (ramp rate: 0.1°C/9 sec.).

e) After adding 3 μl T4 DNA ligase (1 μg/μl), incubation at 16°C overnight.

9. Synthesis of the 2nd difference product
By adding 100 ml water, the ligation batch from step 8e) was diluted to a concentration of 1.25 ng/μl. 40 μl of this dilution (50 ng) were mixed with 80 μl driver DNA (see item 4.) and treated again according to steps 6. to 8. When the oligonucleotide adapters (step 8.) were exchanged, the J-Bgl oligonucleotides were then ligated to the newly formed difference product 2.

10. Synthesis of the 3rd difference product
The concentration of difference product 2 ligated the J-Bgl oligos was reduced to a concentration of 1 ng/μl. 10 μl of this solution were diluted again with 990 μl water (+ 30 μg yeast RNA), so that the concentration was then 10 pg/μl. The subtractive hybridization was carried out with 100 pg (10 μl) J-ligated difference produce 2 and 40 μg (80 μl) driver DNA from step 4.). As for the rest, the same steps were carried out as in the first and second difference products according to steps 6. to 8. An exception was the PCR

following the MBN reaction (item 7.g) - here only 18 instead of 22 cycles were carried out.

11. Cloning of the 3rd difference product

The 3rd difference product was initially subjected to restriction digestion using DpnII so as to remove the oligonucleotide adapters. The reaction product was then applied to a TAE gel and separated by means of electrophoresis. The separated DNA bands were cut out of the gel, the DNA was eluted and cloned into a vector (pBS Not) cut by BamHI.

12. Characterization of the difference products

In order to confirm that the cloned DNA fragments were not method artifacts but sequences which were actually included in the investigated DNA representations, Southern analyses were carried out in which the investigated cDNA representations were hybridized with the radioactively labeled cloning products.

Thereafter, those DNA fragments which had proved to be "real" difference products in the Southern analysis were investigated by means of Northern hybridizations: RNAs were blotted from the investigated tissues ((whn +/_) skin cDNA and (whn -/-) skin cDNA) and hybridized with the radioactively labeled cloning products. By this, the differential expression of these sequences was confirmed in the investigated tissues. An analysis of the sequences led to the result that the Ha3 gene is not expressed in nu/nu mice (mice suffering from alopecia) (FIGURE 1).

B. Example 2: Expression of Hair Keratin and Whn Genes in Normal Mice and Mice Suffering from Alopecia

RNA was isolated from the skin of differently old normal (whn +/+) and nude (whn -/-) mice, separated electrophoretically in agarose gels, transferred to filters and hybridized with gene-specific probes.

The employed probes were as follows:

mHa1: nucleotides 1331 - 1551; Gene Bank, accession No. M27734

mHa3: nucleotides 1007 - 1024; Gene Bank, accession No. X75650

mHa4: nucleotides 1303 - 1542, cf. Bertolino, A.P. et al., J. Invest. Dermatol. 94, (1990), 297 - 303

whn: nucleotides 1141 - 1374; Gene Bank, accession No. X81593

It showed that hair keratin genes and whn genes in mice suffering from alopecia are not expressed and expressed only slightly, respectively.

C. Example 3: Detection of the Expression Induction of the Ha3 Gene by the Gene Product of the Whn Gene

A whn gene tagged at the N-terminal epitope was inserted in the expression vector pTRE (Clontech). The resulting DNA construct was used for a transient transfection of the HeLa Tet-On cell line (Clontech) by means of the calcium phosphate coprecipitation method. The cells were treated with 5 μg/ml docycline directly afterwards. 1 mM sodium butyrate was added 24 h later. The cells were harvested 48 h after the transfection and subjected to a RT-PCR method. The primers used in the PCR method were as follows:

hHa3:

5' -CTGATCACCAACGTGGAGTC-3',

5' -TACCCAAAGGTGTTGCAAGG-3'.

The PCR method included 35 - 40 cycles each of 30 sec. at 95°C, of 30 sec. at 58°C and of 1 min. at 72°C.

It showed that an expression of the Ha3 gene was induced by the expression of the whn gene. Parallel controls in which no transfection was effected by means of the whn gene, did not result in an induction of the Ha3 gene expression.

D. Example 4: Preparation of the System According to the Invention

A BAC clone referred to as BAC whn, which comprises the entire whn gene of a mouse, was isolated from a BAC library of the company of Genome System (St. Louis, Missouri, U.S.A.) (Schorpp *et al.*, 1997, *Immunogenetics* 46:509-515).

In addition, a shuttle vector referred to as pMBO96-whn-GFP was used, which included the mouse whn gene which contained the reporter gene GFP in exon 3 (Nehls *et al.*, 1996, *Science* 272:886-889).

BAC-whn was used to transform the $recA^+$ E. coli strain CBTS. The transformation was carried out by means of electroporation. Clones were isolated and transformed by means of pMB096-whn-GFP using electroporation. A homologous recombination was made between the BAC clone and the shuttle vector within the range of the whn gene so as to obtain a vector referred to as BAC-whn-GFP. It included the receptor gene GFP in the whn gene.

BAC-whn-GFP was used for the transfection of COS cells. The transfection was carried out by means of the calcium phosphate coprecipitation method. COS cells were obtained which coded for a fusion gene from whn and GFP.

It showed that these cells were suited to identify substances which could induce the gene expression of whn. Such substances were suited to inhibit alopecia.

All references cited within the body of the instant specification are hereby incorporated by reference in their entirety.

CLAIMS

WHAT IS CLAIMED:

1. A process inhibiting alopecia, comprising the increase in the cellular amount of hair keratins.

2. The process according to claim 1, wherein hair keratins are added to the cells.

3. The process according to claim 2, wherein the hair keratins are present in the form of DNA expressing the same.

4. The process according to any one of claims 1 to 3, wherein the gene expression of substances activating hair keratins are added to the cells.

5. The process according to claim 4, wherein the substances are present in the form of DNA expressing the same.

6. The process according to any one of claims 1 to 5, wherein the hair keratins comprise Ha1, Ha2, Ha3, and Ha4.

7. The process according to claim 4 or 5, wherein the substances comprise the gene product of the whn gene and/or the expression of substances activating the whn gene.

8. A system of identifying alopecia-inhibiting substances, comprising the increase in the cellular amount of hair keratins and/or of substances activating the gene expression thereof.

9. The system according to claim 8, wherein the system comprises cells in which one or several expressing hair keratin genes are present in fused form with a reporter gene.

10. The system according to claim 8 or 9, wherein the hair keratins comprise Ha1, Ha2, Ha3, and Ha4.

11. The system according to any one of claim 8 to 10, wherein the system comprises cells in which one or several expressible substances activating the gene expression of hair keratins are present in fused form with the reporter gene.

12. The system according to any one of claims 8 to 11, wherein the substances comprise a gene product of the whn gene.

13. The system according to any one of claims 9 to 12, wherein the reporter gene codes for an enzyme.

14. The system according to any one of claim 9 to 12, wherein the reporter gene codes for a fluorescent protein.

15. The system according to any one of claims 9 to 14, wherein the fusion genes are present in extrachromosomal form.

16. The system according to any one of claims 9 to 14, wherein the fusion genes are integrated in the cell genome.

17. The system to any one of claims 9 to 16, which also comprises substances for the detection of the expressed hair keratins and/or of substances activating the gene expression thereof and fusion genes, respectively.

CLAIMS AS AMENDED IN PCT

1. A process for inhibiting alopecia, comprising the increase in the cellular amount of hair keratins.

2. The process according to claim 1, wherein hair keratins are added to the cells.

3. The process according to claim 2, wherein the hair keratins are present in the form of DNA expressing the same.

4. The process according to any one of claims 1 to 3, wherein the gene expression of substances activating hair keratins are added to the cells.

5. The process according to claim 4, wherein the substances are present in the form of DNA expressing the same.

6. The process according to any one of claims 1 to 5, wherein the hair keratins comprise Ha2, Ha2, Ha3 and Ha4.

7. The process according to claim 4 or 5, wherein the substances comprise the gene product of the whn gene and/or the expression of substances activating the whn gene.

8. A process of identifying alopecia-inhibiting substances, in which the increase in the cellular amount is determined by hair keratins and/or substances activating the gene expression thereof.

9. The process according to claim 8, wherein cells are used in which one or several expressing hair keratin genes are present in fused form with a reporter gene.

10. The process according to claim 8 or 9, wherein the hair keratins comprise Ha1, Ha2, Ha3, and Ha4.

11. The process according to any one of claims 8 to 10, wherein cells are used in which one or several expressible substances activating the gene expression of hair keratins are present in fused form with the reporter gene.

12. The process according to any one of claims 8 to 11, wherein the substances comprise a gene product of the whn gene.

13. The process according to any one of claims 9 to 12, wherein the reporter gene codes for an enzyme.

14. The process according to any one of claims 9 to 12, wherein the reporter gene codes for a fluorescent protein.

15. The process according to any one of claims 9 to 14, wherein the fusion genes are present in extrachromosomal form.

16. The process according to any one of claims 9 to 14, wherein the fusion genes are integrated in the cell genome.

17. The process according to any one of claims 9 to 16, which also uses substances for the detection of the expressed hair keratins and/or of substances activating the gene expression thereof and/or the fusion genes.

ABSTRACT

The present invention relates to a process for inhibiting alopecia, comprising the increase in the cellular amounts of hair keratins and a system of identifying substances inhibiting alopecia.

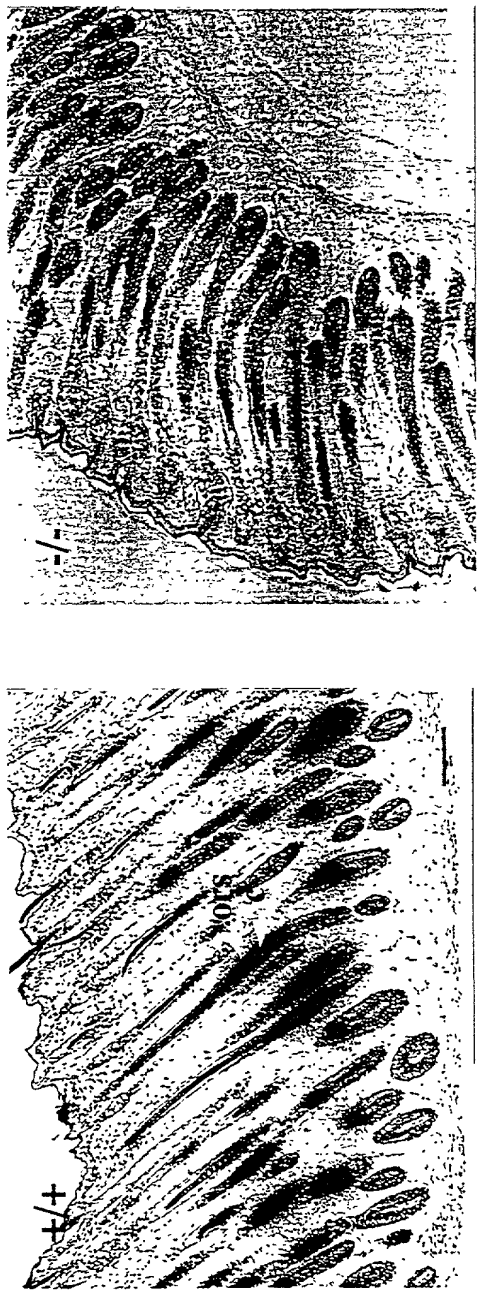

Fig. 1

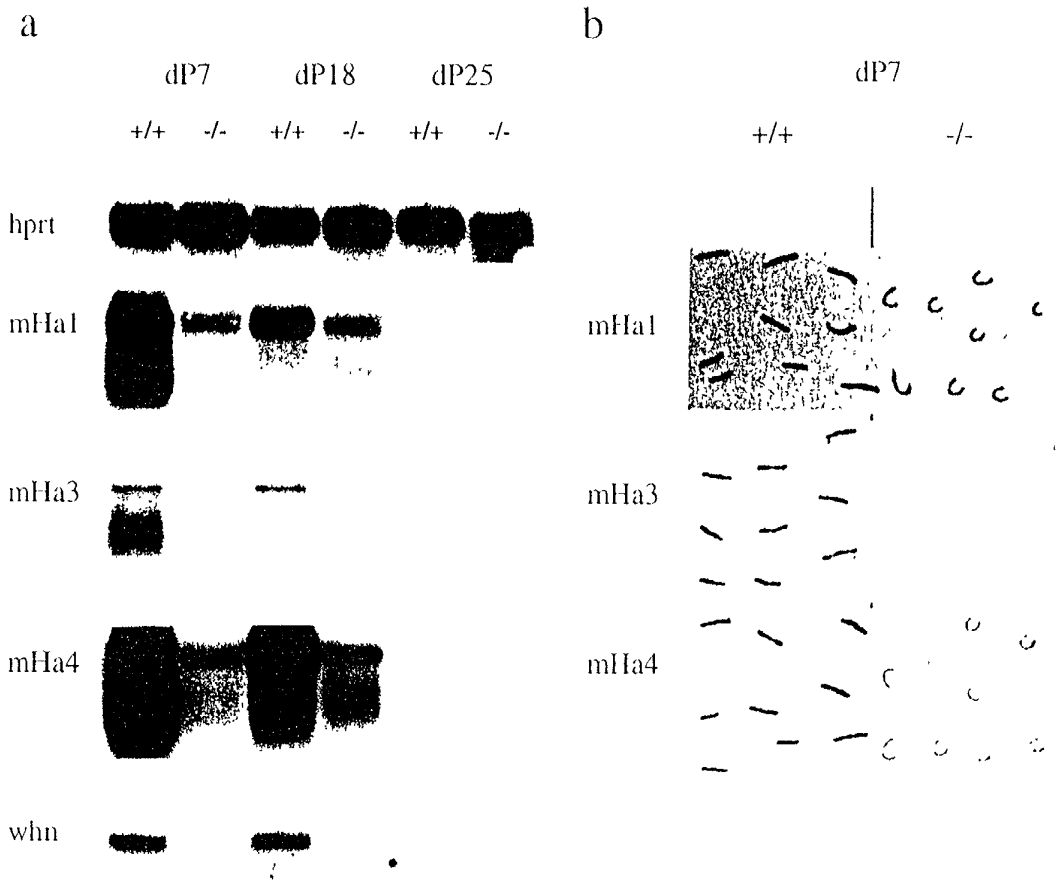

Fig. 2

Fig. 3

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

INHIBITION OF ALOPECIA

This is a national phase filing of the Application No. PCT/DE99/02185, which was filed with the Patent Corporation Treaty on 13 July 1999, and is entitled to priority of the German Patent Application 198 31 043.9, filed 13 July 1998.

I. FIELD OF THE INVENTION [Inhibition of Alopecia]

The present invention relates to a process for inhibiting alopecia and a system of identifying alopecia-inhibiting substances.

II. BACKGROUND OF THE INVENTION

Alopecia is a wide-spread hair disease which may result in the complete loss of the hair. The causes of alopecia are not known. In so far it is not possible to influence this disease in well-calculated fashion.

Therefore, it is the object of the present invention to provide a product by means of which this can be achieved.

According to the invention, this is achieved by the subject matters defined in the claims.

III. SUMMARY OF THE INVENTION

The present invention relates to a process for inhibiting alopecia, comprising the increase in the cellular amounts of hair keratins and a system of identifying substances inhibiting alopecia.

IV. BRIEF DESCRIPTION OF THE DRAWINGS

FIGURE 1 shows an *in situ* RNA hybridization using a probe for mHa3 in normal (whn + / +) and mutant (whn - / -) mice. The transcripts for mHa3 (perceptible as brown

silver grains) cannot be detected in hair follicles of nude mice. The line corresponds to 100 μm.

FIGURE 2 shows the expression of whn and hair keratins in the hair follicle of a mouse.

FIGURE 2A: Northern filter hybridization with RNA from the total skin of normal mice (whn + / +) and nude mice (whn - / -) by means of probes for hprt and whn genes as well as Ha1, Ha3, Ha4 genes at three times following the birth dP7, 7 days after the birth, etc.).

FIGURE 2B: *In situ* RNA hybridization in the skin from normal (whn + / +) and nude mice (whn - / -) with probes for Ha1, Ha3, and Ha4 genes. An autoradiogram of skin cuts on day 7 afer the birth is shown.

FIGURE 3 shows the control of keratin gene expression. HeLa cells were transiently transfected with a whn expression construct (+), and the presence of Ha3-specific mRNA was detected by means of RT-PCR. The molecular weight markers are given in bp.

V. DETAILED DESCRIPTION OF THE INVENTION

It is the object of the present invention to provide a product by means of which this can be achieved.

The present invention is based on the applicant's findings that certain forms of alopecia are based on an unbalanced keratinization of the hair. Furthermore, he has found that in the case of alopecia the mRNA of various genes is lacking, *e.g.*, that of the Ha3 gene, or underrepresented, *e.g.*, those of Ha1, Ha2, and Ha4 genes [(cg. figs.](FIGURES 1 and 2). The gene products of Ha1, Ha2, Ha3, and Ha4 genes are hair keratins. The applicant has found that the expression of the Ha3 gene is controlled by a gene product of the whn gene. In particular, he has found that the expression of the Ha2 gene can be induced by the expression of the whn gene [(cf. fig.](FIGURE 3). He has also found that the expression of other hair keratin genes is essentially influenced by the gene product of the whn gene. The applicant has also found that the expression of the whn gene varies in the course of the hair cycle. In particular, he has found that the whn expression in the telogen of the hair cycle drops to no longer detectable levels. In addition, he has discovered that the whn gene can be

transcribed by two promoters. The applicant has obtained his findings by means of [naked] nude mice and HeLa cells.

According to the invention the applicant's findings are used for a process for inhibiting alopecia, which comprises the increase in the cellular amount of hair keratins.

The expression "increase in the cellular amount of hair keratins" refers to the fact that the amount of one or several hair keratins, particularly of Ha1, H2, Ha3 and Ha4, which may be present in small amount or not al all, is increased in cells. This can be achieved by common methods and substances, respectively. For example, one or several hair keratins, particularly Ha1, Ha2, Ha3 and Ha4, may be added to the cells as such or in the form of DNA encoding the same. The DNA may be present in common expression vectors. It is also possible to add substances which activate the expression of one or several hair keratins, particularly of Ha1, Ha2, Ha3 and Ha4. Such substances are, *e.g.*, the gene product of the whn gene or a DNA encoding the same. It may be present in common expression vectors. Moreover, substances may be added which activate the expression of the whn gene. They may also be present as such or in the form of DNA encoding the same, it being possible for the latter to be also present in common expression vectors. The expression "cells" comprises cells of any kind and origin. In addition, it comprises tissues and organisms, particularly animals and human beings.

Substances inhibiting alopecia can be administered as usual, preferably locally. The substances may also be present in common formulations. If the substances are administered locally, *e.g.*, creams, ointments, shampoos and hair tonics will be suitable. The substances may also be present as particles which are easily absorbed. Examples of such particles are liposomes. A person skilled in the art knows processes to discover the suitable formulations and forms of administration, respectively, for the individual substances.

A further subject matter of the present invention relates to a system of identifying substances which are suited to inhibit alopecia. Such a system comprises the increase in the cellular amount of hair keratins and/or substances activating the gene expression thereof. In particular, the system comprises animals or cells, cells being preferred, in which one or several expressible hair keratin genes and/or one or several expressible genes, whose gene products activate the gene expression of hair keratins, are present each in fused form with a reporter gene. The hair keratin genes may be particularly those of Ha1, Ha2, Ha3 and Ha4.

Moreover, it is favorable for the substance activating the gene expression of hair keratins to be a gene product of the whn gene. In addition, the above genes may have a wild type sequence or a modified sequence, it being possible for the latter to differ from the wild type sequence by one or several base pairs. The differences may exist in the form of additions, deletions, substitutions and/or inversions of base pairs. Besides, an above reporter gene may be any gene, particularly it may code for an enzyme, *e.g.*, alkaline phosphatase, or a fluorescent protein, *e.g.*, GFP. The fusion genes may also be available in extrachromosomal fashion or in the cell genome, particularly in place of one or both [allels] alleles of hair keratins and/or the genes whose gene products activate the expression of hair keratins. Besides, the system may contain substances which are suited to detect the expressed hair keratins and/or substances activating the gene expression thereof and the fusion genes, respectively. Such substances may be suited for the detection on a nucleic acid level and protein level, respectively.

By means of the present invention it is possible to inhibit alopecia. It is also possible to diagnose alopecia by determining, *e.g.*, the gene expression of hair keratins and/or substances which activate it. Moreover, it is possible to discover substances which are adapted to inhibit alopecia. For this purpose, a system is provided which is suited for the rapid and reliable screening of the most varying substances. Thus, the present invention provides products serving for diagnosing and treating a wide-spread hair disease.

[Brief description of the drawings:

Fig. 1 shows an in situ RNA hybridization using a probe for mHa3 in normal (whn + / +) and mutant (whn - / -) mice. The transcripts for mHa3 (perceptible as brown silver grains) cannot be detected in hair follicles of naked mice. The line corresponds to 100 μm.

Fig. 2 shows the expression of whn and hair keratins in the hair follicle of a mouse.

A. Northern filter hybridization with RNA from the total skin of normal mice (whn + / +) and naked mice (whn - / -) by means of probes for hprt and whn genes as well as Ha1, Ha3, Ha4 genes at three times following the birth dP7, 7 days after the birth, etc.).

B. In situ RNA hybridization in the skin from normal (whn + / +) and naked mice (whn - / -) with probes for Ha1, Ha3, and Ha4 genes. An autoradiogram of skin cuts on day 7 afer the birth is shown.

Fig. 3 shows the control of keratin gene expression. HeLa cells were transiently transfected with a whn expression construct (+), and the presence of Ha3-specific mRNA was detected by means of RT-PCR. The molecular weight markers are given in bp]

The below examples explain the invention in more detail. The following preparations and examples are given to enable those skilled in the art to more clearly understand and to practice the present invention.

The present invention [is explained by the below examples:], however, is not limited in scope by the exemplified embodiments, which are intended as illustrations of single aspects of the invention only, and methods which are functionally equivalent are within the scope of the invention. Indeed, various modifications of the invention in addition to those described herein will become apparent to those skilled in the art from the foregoing description and accompanying drawings. Such modifications are intended to fall within the scope of the appended claims.

VI. EXAMPLES

A. Example 1: Detection of the [loss] Loss of [expression] Expression of the Ha3 [gene] Gene in [mice suffering] Mice Suffering from [alopecia] Alopecia

The "Representational Difference Analysis" (RDA) method was carried out. This method comprises the isolation of mRNA from skin cells of (whn + / +) mice and (whn - / -) mice (mice suffering from alopecia and having no expression of the whn gene), respectively, the transcription of mRNA into cDNA, and the differentiation of the cDNA, thereby identifying the one underexpressed and overexpressed, respectively, in (whn - / -) mice.

A) Sequence of the Oligonucleotide Adapters [oligonucleotide adapters

The following oligonucleotide adapter pairs were required for RDA:]

The following oligonucleotide adapter pairs were required for RDA:

R-Bgl-12: 5' -GATCTGCGGTGA-3'

R-Bgl-24: 5' -AGCACTCTCCAGCCTCTCACCGCA-3'

R-Bgl-12: 5' -GATCTGTTCATG-3'

R-Bgl-24: 5' -ACCGACGTCGACTATCCATGAACA-3'

R-Bgl-12: 5' -GATCTTCCCTCG-3'

R-Bgl-24: 5' -AGGCAACTGTGCTATCCGAGGGAA-3'

B) Production of [poly] Poly A-[mRNA] mRna from [tissues] Tissues to [be compared] Be Compared with [one another] One Another

First, RNA was obtained from the skin of (whn + / +) mice and (whn - / -) mice, respectively, according to the "single step RNA extraction" method (Chomczynski and Sacchi, 1987). The poly A-mRNA fractions from both RNA populations were then isolated by means of dynabeads oligo(dT) according to the corresponding protocol from the company of [Dynal] Dynal.

C) Synthesis of [double] Double-stranded cDNA

The "ribo clone DNA synthesis kit" from the company of Promega was used for the synthesis of double-stranded (whn + / +) cDNA and (whn - / -) cDNA, respectively. 4 μg poly A-mRNA were used each to obtain about 2 μg cDNA.

D) Difference [analysis] Analysis

1. Restriction digestion of the double-stranded cDNAs.

   a) About 2 μg of each cDNA were digested in a 100 μl reaction batch by the restriction endonuclease DphII at 37°C for 2 h.

   b) The reaction solutions were then extracted twice using a phenol/chloroform mixture (1:1) and once using 100% chloroform.

   c) The DNA included in the aqueous phases of the two reaction batches was mixed with 2 μg glycogen, 50 μl 10 M ammonium acetate, and 650 μl 100% ethanol each and precipitated on ice for 20 min. Following 14 minutes of centrifugation at 4°C and 14,000 rpm, the supernatant was discarded and the DNA pellet was washed with 70% ethanol. After another centrifugation and removal of the alcoholic phase, the dried DNA was resuspened in 20 μl TE buffer.

2. Litigation of the cDNAs to the R-Bgl oligonucleotide adapter pair

   a) A reaction vessel collected the following:

   20 μl cut cDNA (total reaction batch from item D)1c)
   8 μg R-Bgl-24
   4 μg R-Bgl-12
   6 μl 10 x ligase buffer
   x μl water
   57 μl final volume

b) The reaction batch was heated in a thermocycler (Peltier Thermocycler PTC-200, MJ Research) to 50°C, kept at this temperature for 1 min., and then cooled again down to 10°C in the course of one hour (ramp rate: 0.1°C/9 sec).

c) After adding 3 μl T4 DNA ligase (1 U/μl), the mixture was incubated at 16°C overnight.

3. Synthesis of "representations" of the cDNA populations to be compared with one another

a) In order to generate what is called "representations" of the ligated cDNAs, the volume of the ligation batches from item 2c) was initially completed by adding 140 μl water each to give 200 μl.
Then, 30 reactions of 200 μl each were prepared from this dilute solution per cDNA population (whn + / +) skin and (whn - / -) skin.
The following reactants were added to such a batch one after the other:
143 μl water
20 μl 10x PCR buffer
20 μl 2 nM dNTPs
20 μl 25 nM Mg chloride
2 μl R-Bgl-24 (1 μg/μl)
4 μl dilute ligation batch

b) PCR:
3 min.: 72 °C
addition of 1 μl Taq-DNA polymerase (5 U/μl)
20 x: 5 min. : 95°C
3 min.: 72°C
finally: cooling to 4°C.

c) For preparing the reaction solutions, 4 reaction batches each were collected in a vessel.

Extraction:	2 x with 700 µl phenol/chloroform each (1:1), 1 x with chloroform 100 %;
Precipitation:	addition of 75 µl 3 M Na acetate solution (pH 5.3) and 800 µl 2-propanol to each reaction vessel, 20 min. on ice.
Centrifugation:	14 min., 14,000 rpm, 4°C.

Washing of the DNA pellets with ethanol 70% and resuspension in such an amount of water that a concentration of 0.5 µg/µl resulted.

4. Restriction digestion of the "representations"

a) In order to remove the R-Bgl oligonucleotide adapters, 300 µg of each representation (whn + / +) skin and (whn - / -) skin, repetitively, were subjected to restriction digestion. After adding the following reactants, incubation was carried out at 37°C for 4 hours:

600 µl cDNA representation (0.5 µg/µl)
140 µl 10 x DpnII buffer
100 µl DpnII (10 U/µl)
560 µl water.

b) The restriction digestion batch was distributed to 2 vessels prior to its preparation.

Extraction:	2 x phenol/chloroform (1:1), 1 x chloroform 100 %;
Precipitating:	addition of 70 µl 3 M Na acetate (pH 5.3), 700 µl 2-propanol to each vessel, 20 min. on ice.
Centrifugation:	14 min., 14,000 rpm, 4°C.

The DNA pellet was washed with ethanol 70% and resuspended in such an amount of water that a concentration of 0.5 µg/µl resulted.

The resulting DpnII-digested (whn + / +) skin cDNA representation represented the driver DNA population to be used for the subtractive hybridization.

5. Synthesis of the tester DNA population

a) 20 μg of the (whn - / -) skin cDNA representation digested by DpnII (= tester DNA) was separated in a TAE gel by means of electrophoresis:

40 μl tester DNA (0.5 μg/μl)

50 μl Te buffer

10 μl 10 x loading buffer

were placed on a 1.2% agarose TAE gel. A voltage was applied to the gel until the bromophenol blue component of the loading buffer had migrated about 2 cm.

b) Thereafter, the bands containing the representation DNA were cut out off the gel and eluted by means of the "agarose gel DNA extraction kit" from the company of Boehringer Mannheim.
The DNA extracts were collected, so that a total of 60 μl solution was obtained. The concentration of this solution was evaluated by electrophoresis of 5 μl in a 1% agarose gel.

c) Eventually, the tester DNA was ligated with the J-oligonucleotide pair:

2 μg tester DNA eluate

6 μl 10 x ligase buffer

4 μl J-Bgl-24 (2 μg/μl)

4 μl J-Bgl-12 (1 μg/μl)

x μl water

57 μl final volume

d) Transferring the reaction batch to the thermocycler:
1 min.: 50°C
cooling down to 10°C within 1 h (ramp rate: 0.1°C/9 sec.)

e) The addition of 3 µl T4 DNA ligase (1U/µl) was followed by incubation at 16°C overnight.

f) Adjustment of the concentration of the tester DNA to about 10 ng/µl by the addition of 120 µl water.

6. Subtractive hybridization

a) 80 µl driver DNA (40 µg) from step 4 and 40 µl (0.4 µg) dilute tester DNA from step 5., ligated with J-oligonucleotides, were collected in a reaction vessel and extracted 2 x with phenol/chloroform (1:1) and once with chloroform 100%.

b) Precipitation by adding 30 µl 10 M ammonium acetate, 380 µl ethanol 100%, -70°C for 10 min.
Centrifugation: 14 min., 14,000 rpm, 4°C
Thereafter: 2 x washing the pellet with ethanol 70%, short centrifugation after each wash step; drying of the DNA pellet.

c) The DNA was resuspended in 4 µl EE x3 buffer (30 mM EPPS, pH 8.0 at 20°C (company of Sigma), 3 mM EDTA) - with pipetting off and on for about 2 min., then heated to 37°C for 5 min., shortly vortexed and eventually the solution was collected again at the vessel bottom by centrifugation. Finally, the solution was coated with 35 µl of mineral oil.

d) Transferring the reaction batch to the thermocycler:

5 min.: 98°C,[] cooling down to 67°C and immediate addition of 1 µl 5 M NaCl to the DNA, incubation at 67°C for 20 h.

7. Synthesis of the first difference product

a) After removing the mineral oil as completely as possible, the DNA was diluted step-wise:

[1.]

(1) addition of 8 µl TE (+ 5 µg/µl yeast RNA),

[2.]

(2) addition of 25 µl TE - thereafter thorough mixing,

[3.]

(3) addition of 362 µl TE - vortex.

b) 4 PCRs were prepared for each subtractive hybridization. Per reaction:
127 µl water
20 µl 10 x buffer
20 µl 2 mM dNTPs
5 µl 25 mM Mg chloride
20 µl dilute hybridization solution (from step 7a))

c) PCR program:
3 min.: 72°C
addition of 1 µl Taq DNA polymerase (5 U/µl)
5 min.: 72°C
addition of 2 µl primer J-Bgl-24 (1 µg/µl)
10 x: 1 min.: 95°C
3 min.: 70°C
finally: 10 min.: 72°C, then cooling down to room temperature.

d) The 4 reaction batches were collected in a 1.5 ml vessel.
Extraction: 2 x phenol/chloroform (1:1), 1 x chloroform 100%.

After the addition of 2 µg glycogen carrier:
Precipitation with 75 µl 3 M Na acetate (pH 5.3), 800 µl 2-propanol, 20 min. on ice.
Centrifugation: 14 min., 14,000 rpm, 4°C.
Washing of the DNA pellet with ethanol 70%.
After drying the DNA, resuspension in 40 µl water.

e) 20 µl of the resuspended DNA from d) were subjected to mung bean nuclease digestion (+ MBN):
20 µl DNA
4 µl 10 x mung bean nuclease buffer (company of NEB)
14 µl water
2 µl mung bean nuclease (10 U/µl; company of NEB)
35 min., 30°C.
The reaction was stopped by adding 160 µl of 50 mM Tris-HCl (ph 8.9) and 5 minutes of incubation at 98°C. Thereafter, the vessel was placed on ice up to the next step.

f) During the MBN incubation, 4 further PCRs were prepared on ice:
127 µl water
20 µl 2 mM dNTPs
10 µl 25 mM Mg chloride
2 µl J-Bgl-24 (1 µg/µl)
20 µl MBN-digested DNA.

g) PCR program:
1 min.: 95°C
allowing to cool down to 80°C, addition of 1 µl Taq DNA polymerase (5 U/µl)
18 x: 1 min.: 95°C
3 min.: 70°C

finally: 10 min.: 72°C, allowing to cool down to 4°C.

h) The 4 PCR batches were collected in a vessel

Extraction: 2 x phenol/chloroform (1:1)
1 x chloroform 100%.

Precipitation: 75 µl 3 M Na acetate (pH 5.3), 800 µl 2-propanol, 20 min. on ice.

Centrifugation: 14 min., 14,000 rpm, 4°C.

Washing of the DNA pellet with ethanol 70%.

Resuspension of the DNA in 100 µl water (resulting concentration: 0.5 µg/µl); the resulting solution represented the first difference product.

8. Exchange of the oligonucleotide adapters of the difference product.

a) Removal of the oligonucleotide adapters by restriction digestion using DpnII:

40 µl difference product 1 (0.5 µg/µl)
30 µl 10 x DpnII buffer
15 µl DpnII (U/µl)
215 µl water
37°C for 2 h.

b) Preparation of the reaction batch:

Extraction: 2 x phenol/chloroform (1:1), 1 x chloroform 100%.

Precipitation: 33 µl 3 M Na acetate (pH 5.3), 800 µl ethanol 100%, -20°C for 20 min.

Centrifugation: 14 min., 14,000 rpm, 4°C.

Washing of the pellet in ethanol 70% and resuspension in 40 µl water.

c) Ligation of the difference product to N-Bgl oligonucleotide adapter pair 1 μl of the prepared DNA solution from step b) was diluted with 9 μl water to give a concentration of 50 ng/μl; 4 μl of this solution were used in the following reaction:

4 μl DpnII-digested difference product 1 (200 ng)
6 μl 10 x ligase buffer
2.5 μl N-Bgl-12 (2 μg/μl)
42.5 μl water.

d) After transferring the reaction batch to the thermocycler:
1 min.: 50°C,
allowing to cool down to 10°C within one hour (ramp rate: 0.1°C/9 sec.).

e) After adding 3 μl T4 DNA ligase (1 μg/μl), incubation at 16°C overnight.

9. Synthesis of the 2nd difference product

By adding 100 ml water, the ligation batch from step 8e) was diluted to a concentration of 1.25 ng/μl. 40 μl of this dilution (50 ng) were mixed with 80 μl driver DNA (see item 4.) and treated again according to steps 6. to 8. When the oligonucleotide adapters (step 8.) were exchanged, the J-Bgl oligonucleotides were then ligated to the newly formed difference product 2.

10. Synthesis of the 3rd difference product

The concentration of difference product 2 ligated the J-Bgl oligos was reduced to a concentration of 1 ng/μl. 10 μl of this solution were diluted again with 990 μl water (+ 30 μg yeast RNA), so that the concentration was then 10 pg/μl. The subtractive hybridization was carried out with 100 pg (10 μl) J-ligated difference produce 2 and 40 μg (80 μl) driver DNA from step 4.). As for the rest, the same steps were carried out as in the first and second

difference products according to steps 6. to 8. An exception was the PCR following the MBN reaction (item 7.g) - here only 18 instead of 22 cycles were carried out.

11. Cloning of the 3rd difference product

The 3rd difference product was initially subjected to restriction digestion using DpnII so as to remove the oligonucleotide adapters. The reaction product was then applied to a TAE gel and separated by means of electrophoresis. The separated DNA bands were cut out of the gel, the DNA was eluted and cloned into a vector (pBS Not) cut by BamHI.

12. Characterization of the difference products

In order to confirm that the cloned DNA fragments were not method artifacts but sequences which were actually included in the investigated DNA representations, Southern analyses were carried out in which the investigated cDNA representations were hybridized with the radioactively labeled cloning products.

Thereafter, those DNA fragments which had proved to be "real" difference products in the Southern analysis were investigated by means of Northern hybridizations: RNAs were blotted from the investigated tissues ((whn +/_) skin cDNA and (whn -/-) skin cDNA) and hybridized with the radioactively labeled cloning products. By this, the differential expression of these sequences was confirmed in the investigated tissues. An analysis of the sequences led to the result that the Ha3 gene is not expressed in nu/nu mice (mice suffering from alopecia) [(cf. fig.](FIGURE 1).

B. Example 2: Expression of [hair keratin and whn genes in normal mice and mice suffering from alopecia.] Hair Keratin and Whn Genes in Normal Mice and Mice Suffering from Alopecia

RNA was isolated from the skin of differently old normal (whn +/+) and [naked] nude (whn -/-) mice, separated electrophoretically in agarose gels, transferred to filters and hybridized with gene-specific probes.

The employed probes were as follows:

mHa1: nucleotides 1331 - 1551; Gene Bank, accession No. M27734

mHa3: nucleotides 1007 - 1024; Gene Bank, accession No. X75650

mHa4: nucleotides 1303 - 1542, cf. Bertolino, A.P. et al., J. Invest. Dermatol. 94, (1990), 297 - 303

whn: nucleotides 1141 - 1374; Gene Bank, accession No. X81593

It showed that hair keratin genes and whn genes in mice suffering from alopecia are not expressed and expressed only slightly, respectively.

C. Example 3: Detection of the [expression induction] Expression Induction of the Ha3 [gene] Gene by the [gene product] Gene Product of the [whn gene.] Whn Gene

A whn gene tagged at the N-terminal epitope was inserted in the expression vector pTRE (Clontech). The resulting DNA construct was used for a transient transfection of the HeLa Tet-On cell line (Clontech) by means of the calcium phosphate coprecipitation method. The cells were treated with 5 μg/ml docycline directly afterwards. 1 mM sodium butyrate was added 24 h later. The cells were harvested 48 h after the transfection and subjected to a RT-PCR method. The primers used in the PCR method were as follows:

hHa3:

5' -CTGATCACCAACGTGGAGTC-3',

5' -TACCCAAAGGTGTTGCAAGG-3'.

The PCR method included 35 - 40 cycles each of 30 sec. at 95°C, of 30 sec. at 58°C and of 1 min. at 72°C.

It showed that an expression of the Ha3 gene was induced by the expression of the whn gene. Parallel controls in which no transfection was effected by means of the whn gene, did not result in an induction of the Ha3 gene expression.

D. Example 4: Preparation of the [system according] System According to the [invention] Invention

A BAC clone referred to as BAC whn, which comprises the entire whn gene of a mouse, was isolated from a BAC library of the company of Genome System (St. Louis, Missouri, U.S.A.) [(cf. Schorpp, M.](Schorpp *et al.*, 1997, *Immunogenetics* 46[, (1997),]:509-515).

In addition, a shuttle vector referred to as pMBO96-whn-GFP was used, which included the mouse whn gene which contained the reporter gene GFP in exon 3 [(cf. Nehls, M.](Nehls *et al.*, 1996, *Science* 272[, (1996),]:886-889).

BAC-whn was used to transform the $recA^+$ E. coli strain CBTS. The transformation was carried out by means of electroporation. Clones were isolated and transformed by means of pMB096-whn-GFP using electroporation. A homologous recombination was made between the BAC clone and the shuttle vector within the range of the whn gene so as to obtain a vector referred to as BAC-whn-GFP. It included the receptor gene GFP in the whn gene.

BAC-whn-GFP was used for the transfection of COS cells. The transfection was carried out by means of the calcium phosphate coprecipitation method. COS cells were obtained which coded for a fusion gene from whn and GFP.

It showed that these cells were suited to identify substances which could induce the gene expression of whn. Such substances were suited to inhibit alopecia.

All references cited within the body of the instant specification are hereby incorporated by reference in their entirety.

CLAIMS

WHAT IS CLAIMED: [Claim]

1. A process inhibiting alopecia, comprising the increase in the cellular amount of hair keratins.

2. The process according to claim 1, wherein hair keratins are added to the cells.

3. The process according to claim 2, wherein the hair keratins are present in the form of DNA expressing the same.

4. The process according to any one of claims 1 to 3, wherein the gene expression of substances activating hair keratins are added to the cells.

5. The process according to claim 4, wherein the substances are present in the form of DNA expressing the same.

6. The process according to any one of claims 1 to 5, wherein the hair keratins comprise Ha1, Ha2, Ha3, and Ha4.

7. The process according to claim 4 or 5, wherein the substances comprise the gene product of the whn gene and/or the expression of substances activating the whn gene.

8. A system of identifying alopecia-inhibiting substances, comprising the increase in the cellular amount of hair keratins and/or of substances activating the gene expression thereof.

9. The system according to claim 8, wherein the system comprises cells in which one or several expressing hair keratin genes are present in fused form with a reporter gene.

10. The system according to claim 8 or 9, wherein the hair keratins comprise Ha1, Ha2, Ha3, and Ha4.

11. The system according to any one of claim 8 to 10, wherein the system comprises cells in which one or several expressible substances activating the gene expression of hair keratins are present in fused form with the reporter gene.

12. The system according to any one of claims 8 to 11, wherein the substances comprise a gene product of the whn gene.

13. The system according to any one of claims 9 to 12, wherein the reporter gene codes for an enzyme.

14. The system according to any one of claim 9 to 12, wherein the reporter gene codes for a fluorescent protein.

15. The system according to any one of claims 9 to 14, wherein the fusion genes are present in extrachromosomal form.

16. The system according to any one of claims 9 to 14, wherein the fusion genes are integrated in the cell genome.

17. The system to any one of claims 9 to 16, which also comprises substances for the detection of the expressed hair keratins and/or of substances activating the gene expression thereof and fusion genes, respectively.

CLAIMS AS AMENDED IN PCT

1. A process for inhibiting alopecia, comprising the increase in the cellular amount of hair keratins.

2. The process according to claim 1, wherein hair keratins are added to the cells.

3. The process according to claim 2, wherein the hair keratins are present in the form of DNA expressing the same.

4. The process according to any one of claims 1 to 3, wherein the gene expression of substances activating hair keratins are added to the cells.

5. The process according to claim 4, wherein the substances are present in the form of DNA expressing the same.

6. The process according to any one of claims 1 to 5, wherein the hair keratins comprise Ha2, Ha2, Ha3 and Ha4.

7. The process according to claim 4 or 5, wherein the substances comprise the gene product of the whn gene and/or the expression of substances activating the whn gene.

8. A process of identifying alopecia-inhibiting substances, in which the increase in the cellular amount is determined by hair keratins and/or substances activating the gene expression thereof.

9. The process according to claim 8, wherein cells are used in which one or several expressing hair keratin genes are present in fused form with a reporter gene.

10. The process according to claim 8 or 9, wherein the hair keratins comprise Ha1, Ha2, Ha3, and Ha4.

11. The process according to any one of claims 8 to 10, wherein cells are used in which one or several expressible substances activating the gene expression of hair keratins are present in fused form with the reporter gene.

12. The process according to any one of claims 8 to 11, wherein the substances comprise a gene product of the whn gene.

13. The process according to any one of claims 9 to 12, wherein the reporter gene codes for an enzyme.

14. The process according to any one of claims 9 to 12, wherein the reporter gene codes for a fluorescent protein.

15. The process according to any one of claims 9 to 14, wherein the fusion genes are present in extrachromosomal form.

16. The process according to any one of claims 9 to 14, wherein the fusion genes are integrated in the cell genome.

17. The process according to any one of claims 9 to 16, which also uses substances for the detection of the expressed hair keratins and/or of substances activating the gene expression thereof and/or the fusion genes.

ABSTRACT [Abstract of the Disclosure]

The present invention relates to a process for inhibiting alopecia, comprising the increase in the cellular amounts of hair keratins and a system of identifying substances inhibiting alopecia.

Inhibition of Alopecia

The present invention relates to a process for inhibiting alopecia and a system of identifying alopecia-inhibiting substances.

Alopecia is a wide-spread hair disease which may result in the complete loss of the hair. The causes of alopecia are not known. In so far it is not possible to influence this disease in well-calculated fashion.

Therefore, it is the object of the present invention to provide a product by means of which this can be achieved.

According to the invention, this is achieved by the subject matters defined in the claims.

The present invention is based on the applicant's findings that certain forms of alopecia are based on an unbalanced keratinization of the hair. Furthermore, he has found that in the case of alopecia the mRNA of various genes is lacking, e.g. that of the Ha3 gene, or underrepresented, e.g. those of Ha1, Ha2, and Ha4 genes (cf. figs. 1 and 2). The gene products of Ha1, Ha2, Ha3, and Ha4 genes are hair keratins. The applicant has found that the expression of the Ha3 gene is controlled by a gene product of the whn gene. In particular, he has found that the expression of the Ha3 gene can be induced by the expression of the whn gene (cf. fig. 3). He has also found that the expression of other hair keratin genes is essentially influenced by the gene product of the whn gene. The applicant has also found that the expression of the whn gene varies in the course of the hair cycle. In particular, he has found that the whn expression in the telogen of the hair cycle drops to no longer detectable levels. In addition, he has discovered that the whn gene can be transcribed by two promoters. The applicant has obtained his findings by means of naked mice and HeLa cells.

According to the invention the applicant's findings are used for a process for inhibiting alopecia, which comprises the increase in the cellular amount of hair keratins.

The expression "increase in the cellular amount of hair keratins" refers to the fact that the amount of one or several hair keratins, particularly of Ha1, H2, Ha3 and Ha4, which may be present in small amount or not at all, is increased in cells. This can be achieved by common methods and substances, respectively. For example, one or several hair keratins, particularly Ha1, Ha2, Ha3 and Ha4, may be added to the cells as such or in the form of DNA encoding the same. The DNA may be present in common expression vectors. It is also possible to add substances which activate the expression of one or several hair keratins, particularly of Ha1, Ha2, Ha3 and Ha4. Such substances are e.g. the gene product of the whn gene or a DNA encoding the same. It may be present in common expression vectors. Moreover, substances may be added which activate the expression of the whn gene. They may also be present as such or in the form of DNA encoding the same, it being possible for the latter to be also present in common expression vectors. The expression "cells" comprises cells of any kind and origin. In addition, it comprises tissues and organisms, particularly animals and human beings.

Substances inhibiting alopecia can be administered as usual, preferably locally. The substances may also be present in common formulations. If the substances are administered locally, e.g. creams, ointments, shampoos and hair tonics will be suitable. The substances may also be present as particles which are easily absorbed. Examples of such particles are liposomes. A person skilled in the art knows processes to discover the suitable formulations and forms of administration, respectively, for the individual substances.

A further subject matter of the present invention relates to a system of identifying substances which are suited to inhibit alopecia. Such a system comprises the increase in

the cellular amount of hair keratins and/or substances activating the gene expression thereof. In particular, the system comprises animals or cells, cells being preferred, in which one or several expressible hair keratin genes and/or one or several expressible genes, whose gene products activate the gene expression of hair keratins, are present each in fused form with a reporter gene. The hair keratin genes may be particularly those of Ha1, Ha2, Ha3 and Ha4. Moreover, it is favorable for the substance activating the gene expression of hair keratins to be a gene product of the whn gene. In addition, the above genes may have a wild type sequence or a modified sequence, it being possible for the latter to differ from the wild type sequence by one or several base pairs. The differences may exist in the form of additions, deletions, substitutions and/or inversions of base pairs. Besides, an above reporter gene may be any gene, particularly it may code for an enzyme, e.g. alkaline phosphatase, or a fluorescent protein, e.g. GFP. The fusion genes may also be available in extrachromosomal fashion or in the cell genome, particularly in place of one or both allels of hair keratins and/or the genes whose gene products activate the expression of hair keratins. Besides, the system may contain substances which are suited to detect the expressed hair keratins and/or substances activating the gene expression thereof and the fusion genes, respectively. Such substances may be suited for the detection on a nucleic acid level and protein level, respectively.

By means of the present invention it is possible to inhibit alopecia. It is also possible to diagnose alopecia by determining e.g. the gene expression of hair keratins and/or substances which activate it. Moreover, it is possible to discover substances which are adapted to inhibit alopecia. For this purpose, a system is provided which is suited for the rapid and reliable screening of the most varying substances. Thus, the present invention provides products serving for diagnosing and treating a wide-spread hair disease.

Brief description of the drawings:

Fig. 1 shows an *in situ* RNA hybridization using a probe for mHa3 in normal (whn +/+) and mutant (whn -/-) mice. The transcripts for mHa3 (perceptible as brown silver grains) cannot be detected in hair follicles of naked mice. The line corresponds to 100 μm.

Fig. 2 shows the expression of whn and hair keratins in the hair follicle of a mouse.

A. Northern filter hybridization with RNA from the total skin of normal mice (whn +/+) and naked mice (whn -/-) by means of probes for hprt and whn genes as well as Ha1, Ha3, Ha4 genes at three times following the birth dP7, 7 days after the birth, etc.).

B. *In situ* RNA hybridization in the skin from normal (whn +/+) and naked mice (whn -/-) with probes for Ha1, Ha3, and Ha4 genes. An autoradiogram of skin cuts on day 7 after the birth is shown.

Fig. 3 shows the control of keratin gene expression. HeLa cells were transiently transfected with a whn expression construct (+), and the presence of Ha3-specific mRNA was detected by means of RT-PCR. The molecular weight markers are given in bp.

The present invention is explained by the below examples:

Example 1: Detection of the loss of expression of the Ha3 gene in mice suffering from alopecia

The "Representational Difference Analysis" (RDA) method was carried out. This method

comprises the isolation of mRNA from skin cells of (whn +/+) mice and (whn -/-) mice (mice suffering from alopecia and having no expression of the whn gene), respectively, the transcription of mRNA into cDNA, and the differentiation of the cDNA, thereby identifying the one underexpressed and overexpressed, respectively, in (whn -/-) mice.

A) Sequence of the oligonucleotide adapters

The following oligonucleotide adapter pairs were required for RDA:

R-Bgl-12: 5'-GATCTGCGGTGA-3'
R-Bgl-24: 5'-AGCACTCTCCAGCCTCTCACCGCA-3'

R-Bgl-12: 5'-GATCTGTTCATG-3'
R-Bgl-24: 5'-ACCGACGTCGACTATCCATGAACA-3'

R-Bgl-12: 5'-GATCTTCCCTCG-3'
R-Bgl-24: 5'-AGGCAACTGTGCTATCCGAGGGAA-3'

B) Production of poly A-mRNA from tissues to be compared with one another

First, RNA was obtained from the skin of (whn +/+) mice and (whn -/-) mice, respectively, according to the "single step RNA extraction" method (Chomczynski and Sacchi, 1987). The poly A-mRNA fractions from both RNA populations were then isolated by means of dynabeads oligo(dT) according to the corresponding protocol from the company of Dynal.

C) Synthesis of double-stranded cDNA

The "ribo clone cDNA synthesis kit" from the company of Promega was used for the synthesis of double-stranded (whn +/+) cDNA and (whn -/-) cDNA, respectively. 4 µg poly A-mRNA were used each to obtain about 2 µg cDNA.

D) Difference analysis

1. Restriction digestion of the double-stranded cDNAs

a) About 2 µg of each cDNA were digested in a 100 µl reaction batch by the restriction endonuclease DpnII at 37°C for 2 h.

b) The reaction solutions were then extracted twice using a phenol/chloroform mixture (1:1) and once using 100 % chloroform.

c) The DNA included in the aqueous phases of the two reaction batches was mixed with 2 µg glycogen, 50 µl 10 M ammonium acetate, and 650 µl 100 % ethanol each and precipitated on ice for 20 min.

Following 14 minutes of centrifugation at 4°C and 14,000 rpm, the supernatant was discarded and the DNA pellet was washed with 70 % ethanol. After another centrifugation and removal of the alcoholic phase, the dried DNA was resuspended in 20 µl TE buffer.

2. Ligation of the cDNAs to the R-Bgl oligonucleotide adapter pair

a) A reaction vessel collected the following:

20 µl cut cDNA (total reaction batch from item D)1c)
8 µg R-Bgl-24
4 µg R-Bgl-12
6 µl 10 x ligase buffer
x µl water
57 µl final volume

b) The reaction batch was heated in a thermocycler (Peltier Thermocycler PTC-200, MJ Research) to 50°C, kept at this temperature for 1 min., and then cooled again down to 10°C in the course of one hour (ramp rate: 0.1°C/9 sec).

c) After adding 3 µl T4 DNA ligase (1 U/µl), the mixture was incubated at 16°C overnight.

3. Synthesis of "representations" of the cDNA populations to be compared with one another

a) In order to generate what is called "representations" of the ligated cDNAs, the volume of the ligation batches from item 2c) was initially completed by adding 140 µl water each to give 200 µl.

Then, 30 reactions of 200 µl each were prepared from this dilute solution per cDNA population (whn +/+) skin and (whn -/-) skin.

The following reactants were added to such a batch one after the other:
143 µl water
20 µl 10x PCR buffer
20 µl 2 mM dNTPs
10 µl 25 mM Mg chloride
2 µl R-Bgl-24 (1 µg/µl)
4 µl dilute ligation batch

b) PCR:
3 min.: 72°C
addition of 1 µl Taq-DNA polymerase (5 U/µl)
20 x: 5 min.: 95°C
3 min.: 72°C
finally: cooling to 4°C.

c) For preparing the reaction solutions, 4 reaction batches each were collected in a vessel.

Extraction: 2 x with 700 µl phenol/chloroform each (1:1), 1 x with chloroform 100 %;

Precipitation: addition of 75 µl 3 M Na acetate solution (pH 5.3) and 800 µl 2-propanol to each reaction vessel, 20 min. on ice.

Centrifugation: 14 min., 14,000 rpm, 4°C.

Washing of the DNA pellets with ethanol 70 % and resuspension in such an amount of water that a concentration of 0.5 µg/µl resulted.

4. Restriction digestion of the "representations"

a) In order to remove the R-Bgl oligonucleotide adapters, 300 µg of each representation (whn +/+) skin and (whn -/-) skin, respectively, were subjected to restriction digestion. After adding the following reactants, incubation was carried out at 37°C for 4 hours:
600 µl cDNA representation (0.5 µg/µl)
140 µl 10 x DpnII buffer
100 µl DpnII (10 U/µl)
560 µl water.

b) The restriction digestion batch was distributed to 2 vessels prior to its preparation.

Extraction: 2 x phenol/chloroform (1:1), 1 x chloroform 100 %;

Precipitation: addition of 70 µl 3 M Na acetate (pH 5.3), 700 µl 2-propanol to each vessel, 20 min. on ice.

Centrifugation: 14 min., 14,000 rpm, 4°C.

The DNA pellet was washed with ethanol 70 % and resuspended in such an amount of water that a concentration of 0.5 µg/µl resulted.

The resulting DpnII-digested (whn +/+) skin cDNA representation represented the driver DNA population to be used for the subtractive hybridization.

5. Synthesis of the tester DNA population

a) 20 µg of the (whn -/-) skin cDNA representation digested by DpnII (=

tester DNA) was separated in a TAE gel by means of electrophoresis:
40 µl tester DNA (0.5 µg/µl)
50 µl Te buffer
10 µl 10 x loading buffer
were placed on a 1.2 % agarose TAE gel. A voltage was applied to the gel until the bromophenol blue component of the loading buffer had migrated about 2 cm.

b) Thereafter, the bands containing the representation DNA were cut out off the gel and eluted by means of the "agarose gel DNA extraction kit" from the company of Boehringer Mannheim.

The DNA extracts were collected, so that a total of 60 µl solution was obtained. The concentration of this solution was evaluated by electrophoresis of 5 µl in a 1 % agarose gel.

c) Eventually, the tester DNA was ligated with the J-oligonucleotide pair:
2 µg tester DNA eluate
6 µl 10 x ligase buffer
4 µl J-Bgl-24 (2 µg/µl)
4 µl J-Bgl-12 (1 µg/µl)
x µl water
57 µl final volume

d) Transferring the reaction batch to the thermocycler:
1 min.: 50°C
cooling down to 10°C within 1 h (ramp rate: 0.1°C/9 sec.)

e) The addition of 3 µl T4 DNA ligase (1 U/µl) was followed by incubation at 16°C overnight.

f) Adjustment of the concentration of the tester DNA to about 10 ng/µl by the addition of 120 µl water.

6. Subtractive hybridization

a) 80 µl driver DNA (40 µg) from step 4. and 40 µl (0.4 µg) dilute tester DNA from step 5., ligated with J-oligonucleotides, were collected in a reaction vessel and extracted 2 x with phenol/chloroform (1:1) and once with chloroform 100 %.

b) Precipitation by adding 30 µl 10 M ammonium acetate, 380 µl ethanol 100 %, -70°C for 10 min.

Centrifugation:	14 min., 14,000 rpm, 4°C
Thereafter:	2 x washing the pellet with ethanol 70 %, short centrifugation after each wash step; drying of the DNA pellet.

c) The DNA was resuspended in 4 µl EE x3 buffer (30 mM EPPS, pH 8.0 at 20°C (company of Sigma), 3 mM EDTA) - with pipetting off and on for about 2 min., then heated to 37°C for 5 min., shortly vortexed and eventually the solution was collected again at the vessel bottom

by centrifugation. Finally, the solution was coated with 35 µl of mineral oil.

d) Transferring the reaction batch to the thermocycler:

5 min.: 98°C,
cooling down to 67°C and immediate addition of 1 µl 5 M NaCl to the DNA,
incubation at 67°C for 20 h.

7. Synthesis of the first difference product

a) After removing the mineral oil as completely as possible, the DNA was diluted step-wise:
1. addition of 8 µl TE (+ 5 µg/µl yeast RNA),
2. addition of 25 µl TE - thereafter thorough mixing,
3. addition of 362 µl TE - vortex.

b) 4 PCRs were prepared for each subtractive hybridization. Per reaction:
127 µl water
20 µl 10 x buffer
20 µl 2 mM dNTPs
5 µl 25 mM Mg chloride
20 µl dilute hybridization solution (from step 7a))

c) PCR program:
3 min.: 72°C
addition of 1 µl Taq DNA polymerase (5 U/µl)
5 min.: 72°C
addition of 2 µl primer J-Bgl-24 (1 µg/µl)

10 x: 1 min.: 95°C
3 min.: 70°C
finally: 10 min.: 72°C, then cooling down to room temperature.

d) The 4 reaction batches were collected in a 1.5 ml vessel.
Extraction: 2 x phenol/chloroform (1:1), 1 x chloroform 100 %.
After the addition of 2 µg glycogen carrier:
Precipitation with 75 µl 3 M Na acetate (pH 5.3), 800 µl 2-propanol, 20 min. on ice.
Centrifugation: 14 min., 14,000 rpm, 4°C.
Washing of the DNA pellet with ethanol 70%.
After drying the DNA, resuspension in 40 µl water.

e) 20 µl of the resuspended DNA from d) were subjected to mung bean nuclease digestion (= MBN):
20 µl DNA
4 µl 10 x mung bean nuclease buffer (company of NEB)
14 µl water
2 µl mung bean nuclease (10 U/µl; company of NEB)
35 min., 30°C.

The reaction was stopped by adding 160 µl of 50 mM Tris-HCl (pH 8.9) and 5 minutes of incubation at 98°C. Thereafter, the vessel was placed on ice up to the next step.

f) During the MBN incubation, 4 further PCRs were prepared on ice:
127 µl water
20 µl 2 mM dNTPs
10 µl 25 mM Mg chloride
2 µl J-Bgl-24 (1 µg/µl)
20 µl MBN-digested DNA.

g) PCR program:
1 min.: 95°C
allowing to cool down to 80°C, addition of 1 µl Taq DNA polymerase (5 U/µl)
18 x: 1 min.: 95°C
3 min.: 70°C
finally: 10 min.: 72°C, allowing to cool down to 4°C.

h) The 4 PCR batches were collected in a vessel
Extraction: 2 x phenol/chloroform (1:1)
1 x chloroform 100 %.
Precipitation: 75 µl 3 M Na acetate (pH 5.3), 800 µl 2-propanol, 20 min. on ice.

Centrifugation: 14 min., 14,000 rpm, 4°C.
Washing of the DNA pellet with ethanol 70 %.
Resuspension of the DNA in 100 µl water (resulting concentration: 0.5 µg/µl); the resulting solution represented the first difference product.

8. Exchange of the oligonucleotide adapters of the difference product

a) Removal of the oligonucleotide adapters by restriction digestion using DpnII:

40 µl difference product 1 (0.5 µg/µl)
30 µl 10 x DpnII buffer
15 µl DpnII (10 U/µl)
215 µl water
37°C for 2 h.

b) Preparation of the reaction batch:

Extraction: 2 x phenol/chloroform (1:1), 1 x chloroform 100 %.
Precipitation: 33 µl 3 M Na acetate (pH 5.3), 800 µl ethanol 100 %, -20°C for 20 min.
Centrifugation: 14 min., 14,000 rpm, 4°C.
Washing of the pellet in ethanol 70 % and resuspension in 40 µl water.

c) Ligation of the difference product to N-Bgl oligonucleotide adapter pair
1 µl of the prepared DNA solution from step b) was diluted with 9 µl water to give a concentration of 50 ng/µl; 4 µl of this solution were used in the following reaction:
4 µl DpnII-digested difference product 1 (200 ng)
6 µl 10 x ligase buffer
2.5 µl N-Bgl-24 (3.5 µg/µl)
2 µl N-Bgl-12 (2 µg/µl)
42.5 µl water.

d) After transferring the reaction batch to the thermocycler:
1 min.: 50°C,
allowing to cool down to 10°C within one hour (ramp rate: 0.1°C/9 sec.).

e) After adding 3 µl T4 DNA ligase (1 µg/µl), incubation at 16°C overnight.

9. Synthesis of the 2nd difference product

By adding 100 ml water, the ligation batch from step 8e) was diluted to a concentration of 1.25 ng/µl. 40 µl of this dilution (50 ng) were mixed with 80 µl driver DNA (see item 4.) and treated again according to steps 6. to 8. When the oligonucleotide adapters (step 8.) were exchanged, the J-Bgl oligonucleotides were then ligated to the newly formed difference product 2.

10. Synthesis of the 3rd difference product

The concentration of difference product 2 ligated with the J-Bgl oligos was reduced to a concentration of 1 ng/µl. 10 µl of this solution were diluted again with 990 µl water (+ 30 µg yeast RNA), so that the concentration was then 10 pg/µl. The subtractive hybridization was carried out with 100 pg (10 µl) J-ligated difference product 2 and 40 µg (80 µl) driver DNA from step 4.). As for the rest, the same steps were carried out as in the first and second difference products according to steps 6. to 8. An exception was the PCR following the MBN reaction (item 7.g) - here only 18 instead of 22 cycles were carried out.

11. Cloning of the 3rd difference product

The 3rd difference product was initially subjected to restriction digestion using DpnII so as to remove the oligonucleotide adapters. The reaction product was then applied to a TAE gel and separated by means of electrophoresis. The separated DNA bands were cut out off the gel, the DNA was eluted and cloned into a vector (pBS Not) cut by BamHI.

12. Characterization of the difference products

In order to confirm that the cloned DNA fragments were not method artifacts but sequences which were actually included in the investigated DNA representations, Southern analyses were carried out in which the investigated cDNA representations were hybridized with the radioactively labeled cloning products.

Thereafter, those DNA fragments which had proved to be "real" difference products in the Southern analysis were investigated by means of Northern hybridizations: RNAs were blotted from the investigated tissues ((whn +/+) skin cDNA and (whn -/-) skin cDNA) and hybridized with the radioactively labeled cloning products. By this, the differential expression of these sequences was confirmed in the investigated tissues. An analysis of the sequences led to the result that the Ha3 gene is not expressed in nu/nu mice (mice suffering from alopecia) (cf. fig. 1).

Example 2: Expression of hair keratin and whn genes in normal mice and mice suffering from alopecia.

RNA was isolated from the skin of differently old normal (whn +/+) and naked (whn -/-) mice, separated electrophoretically in agarose gels, transferred to filters and hybridized with gene-specific probes.

The employed probes were as follows:

mHa1: nucleotides 1331 - 1551; Gene Bank, accession No. M27734
mHa3: nucleotides 1007 - 1204; Gene Bank, accession No. X75650
mHa4: nucleotides 1303 - 1542, cf. Bertolino, A.P. et al., J. Invest. Dermatol. 94, (1990), 297 - 303
whn: nucleotides 1141 - 1374; Gene Bank, accession No. X81593

It showed that hair keratin genes and whn genes in mice suffering from alopecia are not expressed and expressed only slightly, respectively.

Example 3: Detection of the expression induction of the Ha3 gene by the gene product of the whn gene.

A whn gene tagged at the N-terminal epitope was inserted in the expression vector pTRE (Clontech). The resulting DNA construct was used for a transient transfection of the HeLa Tet-On cell line (Clontech) by means of the calcium phosphate coprecipitation method. The cells were treated with 5 µg/ml docycline directly afterwards. 1 mM sodium butyrate was

added 24 h later. The cells were harvested 48 h after the transfection and subjected to a RT-PCR method. The primers used in the PCR method were as follows:

hHa3:
5'-CTGATCACCAACGTGGAGTC-3',
5'-TACCCAAAGGTGTTGCAAGG-3'.
The PCR method included 35 - 40 cycles each of 30 sec. at 95°C, of 30 sec. at 58°C and of 1 min. at 72°C.

It showed that an expression of the Ha3 gene was induced by the expression of the whn gene. Parallel controls in which no transfection was effected by means of the whn gene, did not result in an induction of the Ha3 gene expression.

Example 4: Preparation of the system according to the invention

A BAC clone referred to as BAC whn, which comprises the entire whn gene of a mouse, was isolated from a BAC library of the company of Genome Systems (St. Louis, Missouri, U.S.A.) (cf. Schorpp, M. et al., Immunogenetics 46, (1997), 509-515).

In addition, a shuttle vector referred to as pMBO96-whn-GFP was used, which included the mouse whn gene which contained the reporter gene GFP in exon 3 (cf. Nehls, M. et al., Science 272, (1996),886-889).

BAC-whn was used to transform the $recA^+$ E. coli strain CBTS. The transformation was carried out by means of electroporation.

Clones were isolated and transformed by means of pMB096-whn-GFP using electroporation. A homologous recombination was made between the BAC clone and the shuttle vector within the range of the whn gene so as to obtain a vector referred to as BAC-whn-GFP. It included the reporter gene GFP in the whn gene.

BAC-whn-GFP was used for the transfection of COS cells. The transfection was carried out by means of the calcium phosphate coprecipitation method. COS cells were obtained which coded for a fusion gene from whn and GFP.

It showed that these cells were suited to identify substances which could induce the gene expression of whn. Such substances were suited to inhibit alopecia.

Amended Claims

1. A process for inhibiting alopecia, comprising the increase in the cellular amount of hair keratins.

2. The process according to claim 1, wherein hair keratins are added to the cells.

3. The process according to claim 2, wherein the hair keratins are present in the form of DNA expressing the same.

4. The process according to any one of claims 1 to 3, wherein the gene expression of substances activating hair keratins are added to the cells.

5. The process according to claim 4, wherein the substances are present in the form of DNA expressing the same.

6. The process according to any one of claims 1 to 5, wherein the hair keratins comprise Ha1, Ha2, Ha3 and Ha4.

7. The process according to claim 4 or 5, wherein the substances comprise the gene product of the whn gene and/or the expression of substances activating the whn gene.

8. A process of identifying alopecia-inhibiting substances, in which the increase in the cellular amount is determined by hair keratins and/or substances activating the gene expression thereof.

9. The process according to claim 8, wherein cells are used in which one or several expressing hair keratin genes are present in fused form with a reporter gene.

10. The process according to claim 8 or 9, wherein the hair keratins comprise Ha1, Ha2, Ha3, and Ha4.

11. The process according to any one of claims 8 to 10, wherein cells are used in which one or several expressible substances activating the gene expression of hair keratins are present in fused form with the reporter gene.

12. The process according to any one of claims 8 to 11, wherein the substances comprise a gene product of the whn gene.

13. The process according to any one of claims 9 to 12, wherein the reporter gene codes for an enzyme.

14. The process according to any one of claims 9 to 12, wherein the reporter gene codes for a fluorescent protein.

15. The process according to any one of claims 9 to 14, wherein the fusion genes are present in extrachromosomal form.

16. The process according to any one of claims 9 to 14, wherein the fusion genes are integrated in the cell genome.

17. The process according to any one of claims 9 to 16, which also uses substances for the detection of the expressed hair keratins and/or of substances activating the gene expression thereof and/or the fusion genes.

Abstract of the Disclosure

The present invention relates to a process for inhibiting alopecia, comprising the increase in the cellular amounts of hair keratins and a system of identifying substances inhibiting alopecia.

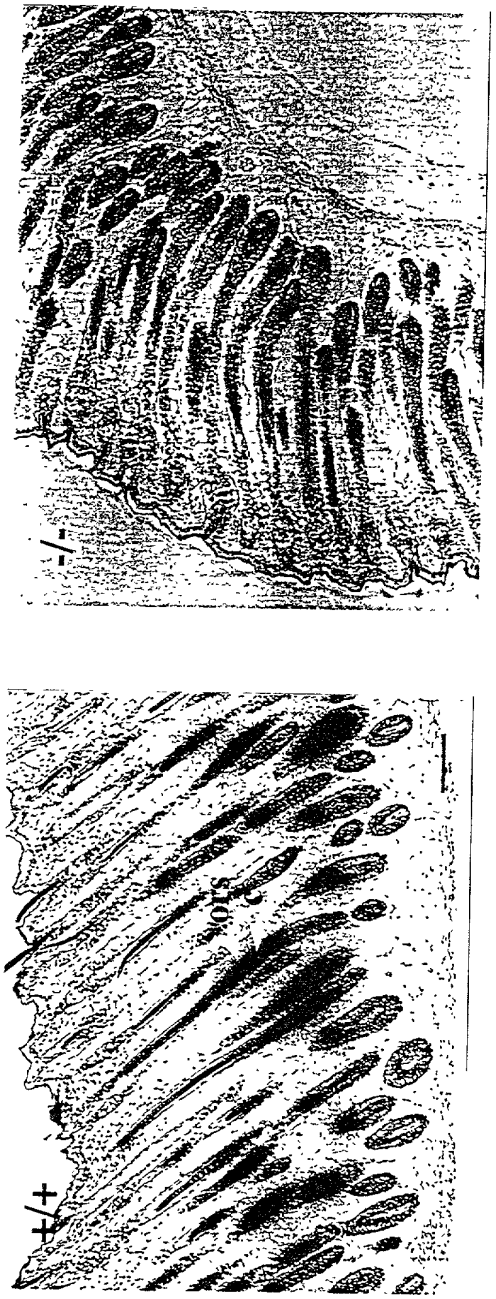

Fig. 1

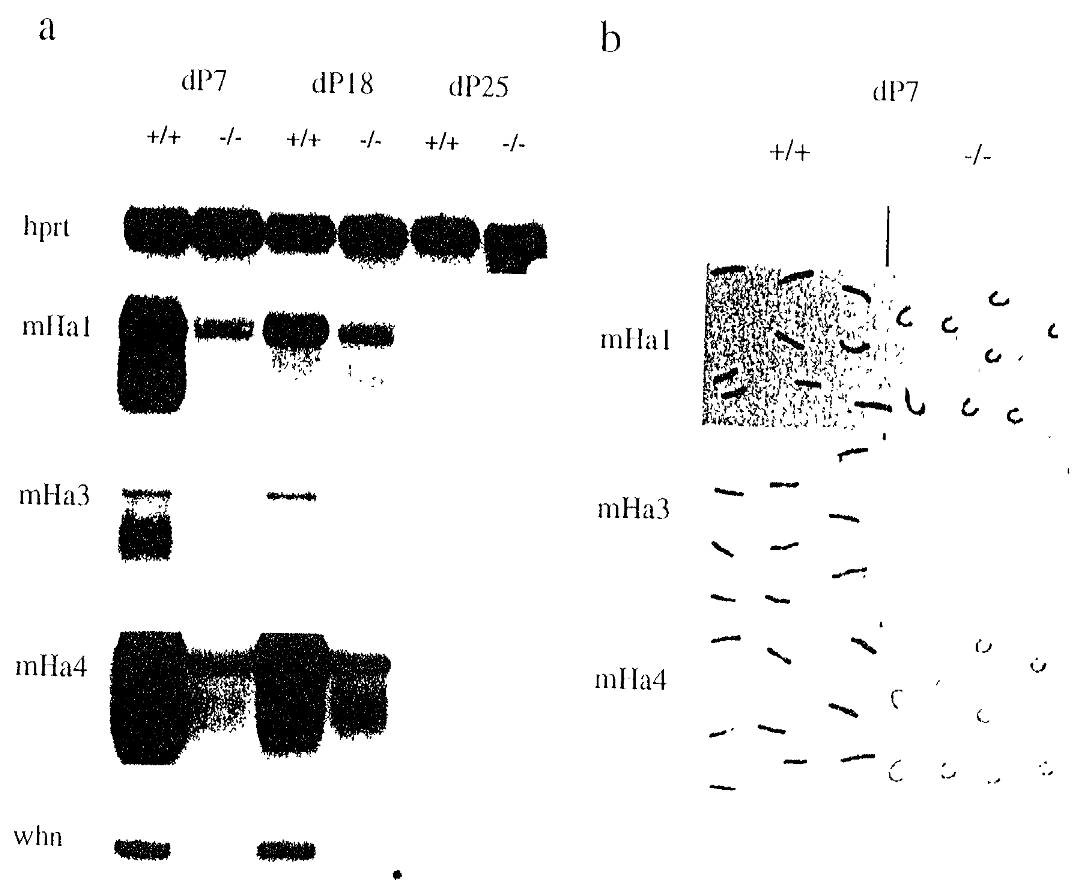

Fig. 2

Fig. 3

DECLARATION FOR NON-PROVISIONAL PATENT APPLICATION*

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below at 201 et seq. beneath my name.

I believe I am the original, first and sole inventor if only one name is listed at 201 below, or an original, first and joint inventor if plural names are listed at 201 et seq. below, of the subject matter which is claimed and for which a patent is sought on the invention entitled

INHIBITION OF ALOPECIA

and for which a patent application:
☒ is attached hereto and includes amendment(s) filed on *(if applicable)*
☐ was filed in the United States on as Application No. *(for declaration not accompanying application)*
with amendment(s) filed on *(if applicable)*
☒ was filed as PCT international Application No. PCT/DE99/02185 on 13 July 1999 and was amended under PCT Article 19 on *(if applicable)*

I hereby state that I have reviewed and understand the contents of the above identified application, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information known to me to be material to patentability as defined in Title 37, Code of Federal Regulations, §1.56.

I hereby claim foreign priority benefits under Title 35, United States Code, §119(a)-(d) of any foreign application(s) for patent or inventor's certificate listed below and have also identified below any foreign application for patent or inventor's certificate having a filing date before that of the application on which priority is claimed:

EARLIEST FOREIGN APPLICATION(S), IF ANY, FILED PRIOR TO THE FILING DATE OF THE APPLICATION			
APPLICATION NUMBER	COUNTRY	DATE OF FILING (day, month, year)	PRIORITY CLAIMED
198 31 043.9	Germany	13 July 1998	YES ☒ NO ☐
			YES ☐ NO ☐
			YES ☐ NO ☐

I hereby claim the benefit under Title 35, United States Code, §119(e) of any United States provisional application(s) listed below.

PROVISIONAL APPLICATION NUMBER	FILING DATE

I hereby claim the benefit under Title 35, United States Code, §120 of any United States application(s) listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application in the manner provided by the first paragraph of Title 35, United States Code §112, I acknowledge the duty to disclose information known to me which is material to patentability as defined in Title 37, Code of Federal Regulations, §1.56 which became available between the filing date of the prior application and the national or PCT international filing date of this application:

NON-PROVISIONAL APPLICATION SERIAL NO.	FILING DATE	STATUS		
		PATENTED	PENDING	ABANDONED

* for use only when the application is assigned to a company, partnership or other organization.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

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